REMARKS

Examination of claims 108-185 is reported in the present Office Action. Claims 128-131 and 159-184 were rejected under 35 U.S.C. § 112, second paragraph, claims 108-185 were rejected under 35 U.S.C. § 103(a), and claims 108-185 were provisionally rejected and rejected under the judicially-created Doctrine of Obviousness-type Double patenting. Each of the rejections is addressed as follows.

First, applicants note that the abstract was objected to as containing more than one paragraph. The specification has now been amended to include an abstract that is one paragraph in length. Also, in reply to the Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, applicants enclose herewith a Sequence Listing in compliance with 37 C.F.R. §§ 1.821 through 1.825.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 128-131 and 159-184 were rejected under 35 U.S.C. 112, second paragraph as being indefinite in reciting the phrase "heterogenous cell strain of transfected secondary cells," because the Examiner states that "a strain is not a number of cells, rather it is a pure isolate of a type of cell." This rejection should be withdrawn.

It is well established in the law that an applicant for patent can be his own lexicographer, provided that a claim term at issue is defined in the specification. *Novo*

Nordisk of North America, Inc. v. Genentech, Inc., 77 F.3d 1364, 1369, 37 U.S.P.Q.2d 1773, 1777 (Fed. Cir. 1996). In the present case, the Examiner has objected to applicants' use of the term "heterogeneous cell strain," on the basis that a "cell strain" is a pure isolate of cells, and thus by definition cannot be heterogeneous. However, applicants have defined the term "heterogenous cell strain" in the specification at page 4, lines 16-18 as "a cell strain that is derived from two or more founder cells." Thus, according to applicants' definition, a "cell strain" can indeed be "heterogenous," and this rejection should therefore be withdrawn.

Rejection under 35 U.S.C. § 103(a)

Claims 108-185 were rejected under § 103(a) for obviousness over Osborne et al. (Proc. Natl. Acad. Sci. U.S.A. 85:6851, 1988) or Palmer et al. (Blood 73:438, 1989), in view of Skoultchi (WO 91/06666) and Browne et al. (Cold Spring Harbor Symposia Quant. Biol. LI:693, 1986).

Osborne and Palmer teach infection of human fibroblasts with retroviral vectors encoding purine nucleoside phosphorylase and human clotting factor IX, respectively. Palmer also teaches introduction of the infected cells into rats and mice, in which the cells produce readily detectable levels of circulating factor IX. Browne teaches the sequence of the human erythropoietin (EPO) gene. The Examiner states that it would have been obvious to use the system of Osborne or Palmer to produce EPO, because Osborne and

Palmer teach that such systems can be used to express heterologous proteins *in vitro* and *in vivo*, and Browne teaches that EPO is an important therapeutic protein. The Examiner further cites Skoultchi as teaching that methods in addition to infection by retroviral vectors (*e.g.*, transfection using methods such as electroporation) can be used to introduce heterologous genes into primary cells.

This rejection should be withdrawn. As is noted above, the claims have now been amended to specify that the primary and secondary cells of the claims are transfected with DNA encoding erythropoietin. Neither of Osborne or Palmer teaches transfection of primary or secondary cells with any gene, let alone a gene encoding erythropoietin. As is noted above, these references teach infection of cells using retroviral vectors and, as is discussed further below, use of transfection to introduce a gene encoding a therapeutic product, such as erythropoietin, into cells, as specified in the present claims, would not have been obvious over references teaching retroviral infection of cells, such as Osborne and Palmer. Also as is discussed further below, Skoultchi, although teaching methods of introducing DNA into cells by transfection, does not provide any motivation to transfect primary or secondary cells with a therapeutic gene, such as the erythropoietin gene, as is required by the amended claims. Finally, Browne, which is cited for teaching the cloning of the EPO gene, makes no mention of transfecting primary or secondary cells with this gene.

Turning first to the Osborne and Palmer references, as is noted above, these papers

teach introduction of genes into cells by retroviral infection. This teaching would not have rendered obvious the use of transfection to introduce a gene encoding a therapeutic product, such as erythropoitein, into primary or secondary cells for a therapeutic purpose, because the prior art taught away from carrying out such methods. For example, in a review of gene transfer technology, Anderson (Science 226:401-409, 1984) stated that "retroviral-based vectors appear to be the most promising approach at present for use in humans" (page 402, column 2). With respect to non-viral methods of gene transfer, Anderson stated that "if a chemical technique for gene transfer were used in a protocol designed for humans, the predicted results appear discouraging" (page 405, column 1). Anderson concluded his review by stating that "effective delivery-expression systems are becoming available" and that "these systems are based on treatment of bone marrow cells with retroviral vectors carrying a normal gene" (page 408, column 1). Thus, Anderson teaches away from non-retroviral transfection methods, such as those that are used in the present invention.

Further teaching away from non-retroviral transfection methods is provided by Gilboa *et al.* (Bio/Techniques 4:504-512, 1986). In dismissing non-retroviral approaches to gene transfer, Gilboa stated that:

> Unfortunately, DNA transfection has its limitations. Most significantly, it is a very inefficient means of transferring genes into mammalian cells... In the past several years, a new gene transfer technology has emerged which appears to be superior to the DNA transfection and other previous techniques and which may offer a new approach to the therapy of human genetic diseases. This new technology is known as retroviral-mediated

gene transfer...

Gilboa further teaches that transfection, the "old" method of gene transfer, was evaluated, attempted, and found to have significant limitations in the context of primary (*i.e.*, "normal") cells. The nature of these limitations is elaborated upon by A. Dusty Miller (Blood 76:271-278, 1990) as follows:

> It has been tempting to assume that the immortal fibroblast cell lines familiar to all cell culturists provide a suitable model for gene therapy involving normal skin fibroblasts. However, there are important differences. Normal skin fibroblasts have a limited lifespan in culture and suffer continuous change in culture that may affect their ability to engraft after transplantation. Thus, gene transfer techniques should be efficient to minimize growth in culture, and cell cloning steps are probably not feasible. It is also important to avoid the use of immortalized cells that spontaneously arise during the culture of fibroblasts, especially with mouse cells, as these immortalized cells may behave differently than normal fibroblasts. Furthermore, gene transfer techniques that work well in continuous cell lines may not work well in normal fibroblasts. For example, normal fibroblasts are much more difficult to transfect than continuous cell lines by calcium phosphate precipitation techniques. In addition, the control of gene expression from a given transcriptional unit may be quite different in normal fibroblasts compared with continuous cell lines; thus, adequate gene expression in continuous cell lines may not predict the situation in normal fibroblasts.

An additional article, by Verma (Scientific American November:68-83, 1990), further teaches away from non-retroviral transfer of DNA into cells. Verma characterizes retroviruses as "the most promising gene-delivery systems studied thus far" (page 70, column 1) and, in characterizing gene delivery by non-viral means, states that "the efficiency of gene delivery is dismal" (page 69, column 2). Thus, Verma also teaches away from non-retroviral transfection methods.

Thus, neither of Osborne or Palmer, in teaching retroviral infection of cells, would

have provided motivation to transfect primary or secondary cells with DNA encoding a therapeutic product, such as erythropoietin, as is required by the amended claims. Non-retroviral transfection is not mentioned in these references, and the art taught away from these methods.

This motivation is also not provided by the Skoultchi reference for several reasons. Briefly, the method of Skoultchi, although involving transfection, does not relate to the introduction of a gene encoding a therapeutic product into a cell. In addition, although Skoultchi refers to certain cells as "primary" and "secondary," these terms have different meanings in Skoultchi, as compared to the present application. Indeed, as is discussed further below, the methods and cells of Skoultchi are entirely different from those of the present claims, and would not have rendered the claimed invention obvious.

The present claims require the introduction of a gene encoding a therapeutic product, erythropoietin, into cells. Skoultchi describes introduction of DNA into cells, but this DNA does not encode a therapeutic product. Rather, the DNA introduced into the cells of Skoultchi includes an amplifiable sequence and regulatory regions. These elements are introduced so as to be functionally associated with a so-called target gene within the cells, and it is this target gene that may encode a therapeutic protein. Thus, Skoultchi describes introduction of DNA into cells to facilitate amplification, and thus expression, of an endogenous gene that encodes a protein of interest, not the introduction of DNA that itself encodes such a protein, as is required by the present claims.

Also, the present claims require the transfection of primary or secondary cells which, as defined in the specification, are cells that have been isolated from a vertebrate tissue source or tissue explant, prior to plating or after plating for the first time (primary cells), or such cells at subsequent stages of culturing (secondary cells) (see, *e.g.*, page 13, lines 8-20 of the specification). Use of such cells is not disclosed in Skoultchi. Rather, the so-called "primary" cells of Skoultchi are cells into the genomes of which an amplifiable gene and regulatory regions have been introduced by homologous recombintation, and the "secondary" cells, rather than being descendants of the initial, "primary" cells, are cells into which DNA removed from the "primary" cells is introduced; the "secondary" cells are not the product of the culturing of those "primary" cells. This is made clear throughout the text of Skoultchi. For example, on page 4, lines 20-25, Skoultchi states:

> The chromosomes or DNA of the transformed cells [i.e., the "primary" cells] are then used to transfer the amplifiable region into the genome of secondary expression host cells, where the target region, if not previously amplified sufficiently or at all, is further amplified.

Thus, in teaching introduction of amplifiable genes or regulatory regions into cells, rather than therapeutic genes, and in requiring the transfer of genetic material from a "primary" cell to a "secondary" cell, rather than the use of primary and secondary cells as defined by applicants, Skoultchi has no bearing on the patentability of the present claims. Browne also fails to have any impact on the patentability of the present claims. Browne teaches the gene encoding EPO and the usefulness of recombinant EPO for therapy, but provides

no motivation to transfect this gene into primary or secondary cells, as is required by the present claims.

In conclusion, prior to applicants' invention, there was no suggestion in the art to transfect primary of secondary cells with an EPO gene. Indeed, as is discussed above, the art taught away from making such cells. Accordingly, the rejection under § 103 can now be withdrawn.

Double Patenting

Claims 108-185 were provisionally rejected under the judicially-created Doctrine of Obviousness Type Double Patenting over claims 135-199 of U.S. Serial No. 09/354,883. When the only rejection remaining in a case is a provisional obviousness-type double patenting rejection, an application should be allowed to issue. M.P.E.P. § 822.01. In view of the amendments and remarks provided in this reply, applicants submit that all of the grounds of rejection in this case, other than the provisional obviousness-type double patenting rejection, have been met. Accordingly, the obviousness-type double patenting rejection should be withdrawn and the case allowed to issue.

Claims 108-185 were also rejected under this Doctrine over claims 1-25 of U.S. Patent No. 5,994,127; claims 1-9 of U.S. Patent No. 6,048,524; claims 1-28 of U.S. Patent No. 6,048,729; claims 1-18 of U.S. Patent No. 6,054,288; and claims 1-24 of U.S. Patent

No. 6,063,630. This rejection is being met by the filing of the enclosed terminal disclaimer, which specifies that the term of any patent issuing in the present case will not be longer than the terms of the cited patents. This rejection can thus now be withdrawn.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is requested. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: May 16, 2001

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U.S. Serial No. 09/328,130, Marked-up Version of Amended Claims
Pursuant to 37 C.F.R. § 1.121(c)(1)(ii)

108. (Amended) A transfected primary or secondary cell having stably integrated into its genome:

a) exogenous DNA that encodes erythropoietin, and

b) DNA sequences that direct expression of the exogenous DNA in the primary or secondary cell.

109. (Amended) The transfected primary or secondary cell of claim 108, wherein said cell is selected from the group consisting of fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, blood cells, muscle cells, hepatocytes, and precursors thereof.

110. (Amended) The transfected primary or secondary cell of claim 108, wherein said cell is of mammalian origin.

111. (Amended) The transfected primary or secondary cell of claim 110, wherein said cell is a human cell.

112. (Amended) The transfected primary or secondary cell of claim 108, further comprising DNA encoding a selectable marker.

113. (Amended) The transfected primary or secondary cell of claim 112, wherein said selectable marker is selected from the group consisting of one that confers nutritional auxotrophy, one that confers resistance to a cytotoxic agent, and one that results in expression of a surface protein.

114. (Amended) The transfected primary or secondary cell of claim 108, wherein said cell is selected from the group consisting of:

a) a primary or secondary cell that, prior to comprising said exogenous DNA, does not make or contain erythropoietin;

b) a primary or secondary cell that, prior to comprising said exogenous DNA, makes or contains erythropoietin in less than physiologically normal amounts or in defective form; and

c) a primary or secondary cell that, prior to comprising said exogenous DNA, makes or contains erythropoietin in physiologically normal amounts.

115. (Amended) A transfected primary or secondary cell comprising:

a) exogenous nucleic acid sequences that encode erythropoietin; and

b) nucleic acid sequences that direct expression of the exogenous nucleic acid sequences in the primary or secondary cell,

wherein the nucleic acid sequences of (a) and (b) are present in the cell episomally.

116. (Amended) The transfected primary or secondary cell of claim 115, wherein said cell is selected from the group consisting of fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, blood cells, muscle cells, hepatocytes, and precursors thereof.

117. (Amended) The transfected primary or secondary cell of claim 115, wherein said cell is of mammalian origin.

118. (Amended) The transfected primary or secondary cell of claim 117, wherein said cell is a human cell.

119. (Amended) The transfected primary or secondary cell of claim 115, further comprising nucleic acid sequences encoding a selectable marker.

120. (Amended) The transfected primary or secondary cell of claim 119, wherein said selectable marker is selected from the group consisting of one that confers nutritional auxotrophy, one that confers resistance to a cytotoxic agent, and one that results in expression of a surface protein.

121. (Amended) The transfected primary or secondary cell of claim 115, wherein said cell is selected from the group consisting of:

a) a primary or secondary cell that, prior to comprising said exogenous nucleic acid sequences, does not make or contain erythropoietin;

b) a primary or secondary cell that, prior to comprising said exogenous nucleic acid sequences, makes or contains erythropoietin in less than physiologically normal amounts or in defective form; and

c) a primary or secondary cell that, prior to comprising said exogenous nucleic acid sequences, makes or contains erythropoietin in physiologically normal amounts.

122. (Amended) A clonal cell strain of transfected secondary cells that express exogenous nucleic acid sequences encoding erythropoietin present therein.

123. (Amended) The clonal cell strain of claim 122, wherein the exogenous nucleic acid sequences are stably incorporated into genomic DNA of the transfected secondary cells.

124. (Amended) The clonal cell strain of claim 122, wherein said transfected secondary cells are selected from the group consisting of fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, blood cells, muscle cells, hepatocytes, and precursors thereof.

125. (Amended) The clonal cell strain of claim 122, wherein said transfected secondary cells are of mammalian origin.

126. (Amended) The clonal cell strain of claim 125, wherein said transfected secondary cells are human cells.

127. (Amended) The clonal cell strain of claim 122, wherein the exogenous nucleic acid sequences are present in the transfected secondary cells episomally.

128. (Amended) A heterogenous cell strain of transfected secondary cells having stably incorporated into their genomes:

a) exogenous DNA encoding erythropoietin, and

b) DNA sequences that direct expression of the exogenous DNA in the secondary cells.

129. (Amended) The heterogenous cell strain of claim 128, wherein the transfected secondary cells are selected from the group consisting of fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, blood cells, muscle cells, hepatocytes, and precursors thereof.

130. (Amended) The heterogenous cell strain of claim 128, wherein said transfected secondary cells are of mammalian origin.

131. (Amended) The heterogenous cell strain of claim 130, wherein said transfected secondary cells are human cells.

132. (Amended) A mixture of cells consisting essentially of transfected primary or secondary cells of claim 108 and primary or secondary cells that do not comprise said exogenous DNA.

133. (Amended) A method of producing a clonal cell strain of transfected secondary cells that express exogenous nucleic acid sequences encoding erythropoietin, said method comprising the steps of:

a) providing a mixture of cells comprising primary cells;

b) transfecting [introducing] into primary cells provided in (a) a nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin and nucleic acid sequences that direct expression of the exogenous nucleic acid sequences in the primary cells, thereby producing transfected primary cells that express the exogenous nucleic acid sequences encoding erythropoietin; and

c) culturing a transfected primary cell produced in (b) to produce a clonal cell strain of transfected secondary cells that express the exogenous nucleic acid sequences encoding erythropoietin.

139. (Amended) The method of claim 133, wherein, in step (b), nucleic acid sequences encoding a selectable marker are transfected [introduced] into primary cells provided in (a).

141. (Amended) The method of claim 133, wherein, in step (b), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into primary cells provided in (a) by electroporation to produce at least one primary cell having the exogenous nucleic acid sequences stably integrated into genomic DNA.

143. (Amended) The method of claim 133, wherein, in step (b), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into primary cells provided in (a) by microinjection.

144. (Amended) The method of claim 133, wherein, in step (b), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into primary cells provided in (a) by a

transfection method selected from the group consisting of calcium phosphate precipitation, modified calcium phosphate precipitation, liposome fusion methodologies, receptor mediated transfer, micro-projectile bombardment, and polybrene precipitation.

146. (Amended) A method of producing a clonal cell strain of transfected secondary cells that express exogenous nucleic acid sequences encoding erythropoietin, said method comprising the steps of:

a) providing a mixture of cells comprising primary cells;

b) producing a population of secondary cells from primary cells provided in (a);

c) transfecting [introducing] into secondary cells produced in (b) a nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin and nucleic acid sequences that direct expression of the exogenous nucleic acid sequences in the secondary cells, thereby producing transfected secondary cells that express the exogenous nucleic acid sequences encoding erythropoietin; and

d) culturing a transfected secondary cell produced in (c) to produce a clonal cell strain of transfected secondary cells that express the exogenous nucleic acid sequences encoding erythropoietin.

152. (Amended) The method of claim 146, wherein, in step (c), nucleic acid sequences encoding a selectable marker are transfected [introduced] into secondary cells produced in (b).

154. (Amended) The method of claim 146, wherein, in step (c), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into secondary cells produced in (b) by electroporation to produce at least one secondary cell having the exogenous nucleic acid sequences stably integrated into genomic DNA.

156. (Amended) The method of claim 146, wherein, in step (c), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into secondary cells produced in (b) by microinjection.

157. (Amended) The method of claim 146, wherein, in step (c), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into secondary cells produced in (b) by a transfection method selected from the group consisting of calcium phosphate precipitation, modified calcium phosphate precipitation, liposome fusion methodologies, receptor mediated transfer, micro-projectile bombardment, and polybrene precipitation.

159. (Amended) A method of producing a heterogenous cell strain of transfected secondary cells that express exogenous nucleic acid sequences encoding erythropoietin, said method comprising the steps of:

a) providing a mixture of cells comprising primary cells;

b) transfecting [introducing] into primary cells provided in (a) a nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin and nucleic acid sequences that direct expression of the exogenous nucleic acid sequences in the primary cells, thereby producing a mixture of primary cells that includes transfected primary cells that express the exogenous nucleic acid sequences encoding erythropoietin;

c) culturing the product of (b) to produce a heterogenous cell strain of transfected secondary cells that express the exogenous nucleic acid sequences encoding erythropoietin.

165. (Amended) The method of claim 159, wherein, in step (b), nucleic acid sequences encoding a selectable marker are transfected [introduced] into primary cells provided in (a).

167. (Amended) The method of claim 159, wherein, in step (b), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into primary cells provided in (a) by

electroporation to produce at least one primary cell having the exogenous nucleic acid sequences stably integrated into genomic DNA.

169. (Amended) The method of claim 159, wherein, in step (b), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into primary cells provided in (a) by microinjection.

170. (Amended) The method of claim 159, wherein, in step (b), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into primary cells provided in (a) by a transfection method selected from the group consisting of calcium phosphate precipitation, modified calcium phosphate precipitation, liposome fusion methodologies, receptor mediated transfer, micro-projectile bombardment, and polybrene precipitation.

172. (Amended) A method of producing a heterogenous cell strain of transfected secondary cells that express exogenous nucleic acid sequences encoding erythropoietin, said method comprising the steps of:

a) providing a mixture of cells comprising primary cells;

b) producing a population of secondary cells from primary cells provided in (a);

c) transfecting [introducing] into secondary cells produced in (b) a nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin and nucleic acid sequences that direct expression of the exogenous nucleic acid sequences in secondary cells, thereby producing a mixture of secondary cells that includes transfected secondary cells that express the exogenous nucleic acid sequences encoding erythropoietin;

d) culturing the product of (c) to produce a heterogenous cell strain of transfected secondary cells that express the exogenous nucleic acid sequences encoding erythropoietin.

178. (Amended) The method of claim 172, wherein, in step (c), nucleic acid sequences encoding a selectable marker are transfected [introduced] into secondary cells produced in (b).

180. (Amended) The method of claim 172, wherein, in step (c), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into secondary cells produced in (b) by electroporation to produce at least one secondary cell having the exogenous nucleic acid sequences stably integrated into genomic DNA.

182. (Amended) The method of claim 172, wherein, in step (c), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding

erythropoietin is transfected [introduced] into secondary cells produced in (b) by microinjection.

183. (Amended) The method of claim 172, wherein, in step (c), the nucleic acid molecule construct comprising exogenous nucleic acid sequences encoding erythropoietin is transfected [introduced] into secondary cells produced in (b) by a method selected from the group consisting of calcium phosphate precipitation, modified calcium phosphate precipitation, liposome fusion methodologies, receptor mediated transfer, micro-projectile bombardment, and polybrene precipitation.

BLOOD

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REVIEW ARTICLE

Progress Toward Human Gene Therapy

By A. Dusty Miller

GENE THERAPY is defined as the transfer of genetic material into the cells of an organism to treat disease. The material transferred is on the order of one to a few genes in size. Since many hereditary diseases are caused by defects in single genes, there are many potential applications of this technique to the treatment of human disease (Table 1). In addition, gene therapy may be useful for acquired diseases, such as cancer or infectious disease.

Gene therapy could be aimed at either germ line or somatic cells of a human, but for ethical and practical reasons, only somatic cell therapy is currently being considered. Thus, gene therapy is similar to organ or tissue transplantation for disease treatment, in the sense that tissue transplantation from normal donors also results in the transfer of normal genes into the patient. However, the techniques are fundamentally different, and their successful application depends on the solution of different problems. For transplantation, these problems primarily involve incompatibility of donor tissue with the transplant recipient. In the case of gene therapy, the major problems are achieving efficient gene transfer and persistent gene expression in appropriate somatic cells.

In this review, I will summarize progress toward the use of gene therapy to treat human disease, focusing on different somatic tissues as potential targets for gene transfer.

STRATEGIES

The ideal application of gene therapy would involve precise repair of a defective gene in the cells of an affected organ, or gene replacement therapy. Such specific gene alterations have been accomplished in cultured cells by homologous recombination of added DNA with endogenous sequences. In a relevant example, a defective β-globin gene was repaired after electroporation of normal β-globin gene fragment.[1] However, the frequency of these events was very low, about 1 in a million. Cells in which homologous recombination has occurred can be isolated by using selectable markers to select for cells that have taken up DNA, and to select for homologous recombination in preference to random integration.[2] Using such strategies, the frequency of homologous recombination is up to 1 in 10 clones isolated. While this technique has promise for ultimate application to gene therapy, practical considerations, such as the finite life span of normal somatic cells or the inability to isolate or grow the relevant transplantable cells, presently preclude its use.

An alternative to gene replacement is the addition of genes to correct a disease, or gene addition therapy. This is currently the most practical approach to gene therapy as gene addition can be achieved with high efficiency. Gene addition therapy has an advantage over gene replacement therapy in that cells that do not normally express a particular gene can be engineered to express that gene. For example, blood clotting factors could be made in any somatic cell, making somatic tissues other than the liver suitable targets for clotting factor gene transfer. Alternatively, entirely new genes might be used to treat disease. For example, the spread of human immunodeficiency virus (HIV) might be inhibited by transfer of genes that encode ribozymes capable of degrading HIV RNA.[3] In comparison with gene replacement, a disadvantage of gene addition therapy is the random insertion of genes into the genome. Thus, the inserted genes may be inappropriately expressed or may lead to inappropriate expression of genes near the insertion site.

Currently the preferred method for gene transfer involves the use of retroviral vectors. Retroviral vectors can be made in the absence of replication-competent helper virus by using retrovirus packaging cell lines (Fig 1). Packaging cells produce the viral proteins required for virion formation and for encapsidation of vector RNA into infectious particles, but do not encapsidate or transmit RNAs encoding viral proteins because of alterations in the genes encoding these proteins. Retroviral vectors promote high efficiency gene transfer approaching 100% even in primary cells. They promote the stable insertion of an unrearranged copy of a gene into the host cell genome, ensuring its presence in all progeny of the infected cell. Retroviral vectors with an amphotropic envelope can infect many mammalian species, including humans, and thus can be tested in animal models before use in humans. One disadvantage of retroviral vectors include the potential of generating replication-competent virus during

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0006-4971/90/7602-0002$3.00/0

Table 1. Diseases Caused by Single Gene Defects: Current Targets for Gene Therapy

Hemophilias
Factor IX deficiency
Factor VIII deficiency
Immunodeficiencies
Adenosine deaminase deficiency
Purine nucleoside phosphorylase deficiency
Urea cycle disorders
Ornithine transcarbamylase deficiency
Argininosuccinate synthetase deficiency
Anemias
β-thalassemia
Sickle cell anemia
Emphysema
α_1-antitrypsin deficiency
Lysosomal storage disorders
Glucocerebrosidase deficiency
Other metabolic disorders
Phenylalanine hydroxylase deficiency
Hypoxanthine-guanine phosphoribosyltransferase deficiency

vector production, although this problem has been effectively solved with improved vector and packaging cell design.[4] Another disadvantage is that retroviral vectors currently cannot be made synthetically from purified components and must be produced from living cells, and thus may contain unwanted contaminants that are difficult to remove. In addition, retroviral vectors have a limited capacity, up to about 7 kb, necessitating the use of cDNAs in the case of large genes.

GENE TRANSFER INTO HEMATOPOIETIC CELLS

The hematopoietic system is an obvious target for gene transfer because of well-developed procedures for bone marrow transplantation, the large number and wide distribution of hematopoietic cells, and the existence of many diseases that affect hematopoietic cells. The hierarchical structure of the hematopoietic system dictates the outcome of gene transfer into a particular cell within the hierarchy. Gene transfer into a differentiated cell type will result in expression of the gene in a restricted class of hematopoietic cells for a limited time. In contrast, transfer into a pluripotent stem cell can result in the continued presence of the gene in all hematopoietic lineages for the life of the animal. Thus, most attempts to transfer genes have focused on gene transfer into the stem cells to provide long-term therapy.

Gene transfer into pluripotent hematopoietic stem cells of the mouse has been demonstrated by many research groups. Pluripotent hematopoietic stem cells are defined by their ability to give rise to all cells of the myeloid and lymphoid lineages and by their ability to provide long-term hematopoiesis in animals. The presence and/or expression of the transferred gene for longer than 4 months posttransplantation in myeloid and lymphoid tissues is generally accepted as evidence for stem cell infection. Sequential transplantation has also been used to demonstrate infection of a cell with extensive repopulating capability.[5-8]

In the most general approach to mouse stem cell infection, donor animals are treated with 5-fluorouracil for about 4 days to kill differentiated cells and, presumably, to induce the replication of normally non-dividing stem cells. This is done because retroviral vectors promote gene transfer only in replicating cells.[9] Bone marrow from leg bones is then removed and infected by co-cultivation with virus-producing fibroblasts for 1 to 5 days in the presence of hematopoietic growth factors. When the vector carries a selectable marker, the marrow is often exposed to the selective agent for 2 days to kill uninfected cells. This procedure results in a greater percentage of the repopulating cells having the transferred gene. Then the marrow is injected into lethally irradiated recipient animals or into *W/W*^{v} mutant mice that can be reconstituted in the absence of irradiation due to a stem cell defect.[10]

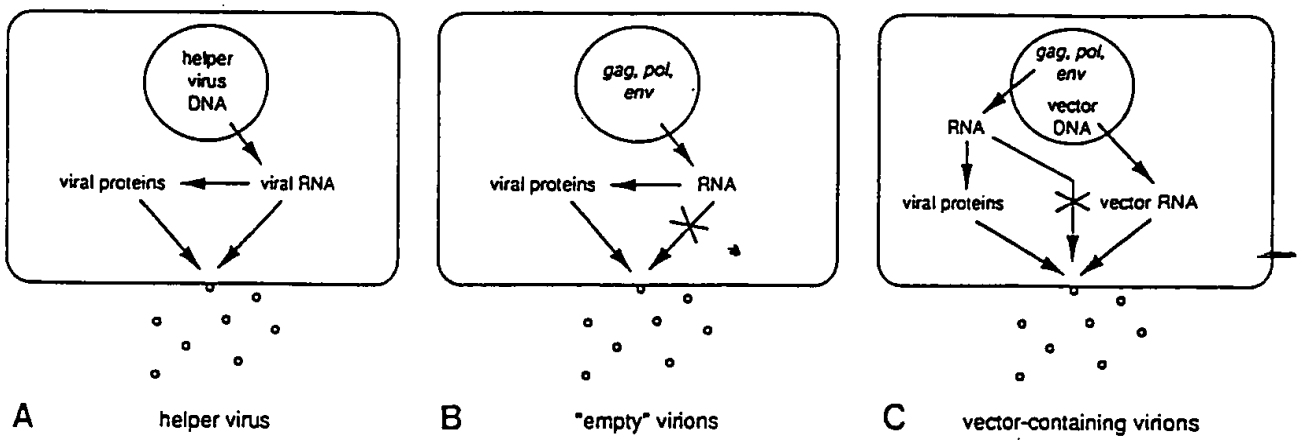

Fig 1. Principles involved in retrovirus packaging cell design. A cell infected with replication-competent helper virus (A), in which viral RNA and proteins combine to form virus that can replicate and spread in infected cells. A packaging cell (B) also synthesizes viral proteins, but the RNAs that encode these proteins are not encapsidated into virions because they have been altered by removal of the retroviral packaging signal. Even if these RNAs are packaged at low frequency, they will not be reverse transcribed or integrated into cells infected with the "empty" virions produced by the packaging cells. Introduction of a retroviral vector into a packaging cell (C) results in encapsidation of vector RNA into infectious virions. Infection of cells with vector-containing virions results in vector transfer but not transfer of genes encoding viral proteins. See reference 4 for further details.

While several early reports demonstrated long-term expression of selectable markers in transplanted hematopoietic cells,[5,10-13] long-term expression of therapeutically relevant genes has been shown only recently. Several reports have shown long-term expression of the human β-globin gene in mice after stem cell infection,[6,7,14] although the highest expression in any mouse was only 1.5% of mouse β-globin levels.[6] On average, when corrected for copy number of the retroviral vector, human β-globin expression was 1% to 2% of that of mouse β-globin in all of the studies, More recently, regulatory elements that strongly enhance β-globin expression in erythroid cells and that are distant from the β-globin gene have been identified.[15] These elements, termed dominant control regions or locus activation regions, were not included in the vectors used, and it seems reasonable that their inclusion will increase β-globin expression from the vectors to therapeutically useful levels. However, inclusion of these elements presents technical problems because the intact elements are large and their inclusion in retroviral vectors inhibits virus production.[16]

Long-term expression of human adenosine deaminase (ADA) has been reported.[17-21] In the earliest study,[17] the results were complicated by the presence of replication-competent helper virus that might artificially increase ADA expression by allowing the ADA vector to spread after bone marrow transplantation. However, later studies have shown that helper virus is not necessary for obtaining long-term ADA expression in mouse hematopoietic cells.[20] In two studies, ADA expression was detected only in red blood or in bone marrow,[20,21] while in the other studies, expression was detected in all hematopoietic tissues analyzed.[17-19] Whether these differences are due to the different vectors used or to differences in experimental systems is not clear. Based on levels of ADA provided by irradiated red blood cells used to treat ADA-deficient patients, about 25% of the normal ADA level in blood would be sufficient for treatment.[22] ADA levels obtained in mice can exceed this level, and thus should be therapeutically useful. However, it is not known whether absolute levels of ADA in specific lymphocyte populations are critical for complete treatment of the patients' immune dysfunction.

A mutant dihydrofolate reductase gene (DHFR) that confers resistance to methotrexate has been expressed in mice and shown to confer a selective advantage to infected cells.[8] Both primary and secondary recipients of infected marrow were partially protected from methotrexate toxicity. This study demonstrates that phenotypic changes can indeed be induced by gene transfer into hematopoietic stem cells. Protection of normal marrow from methotrexate toxicity might allow higher doses of methotrexate in chemotherapy for cancer. Additional examples of gene expression in hematopoietic cells of mice include expression of human argininosuccinate synthetase[23] and human glucocerebrosidase,[24] but long-term expression of these proteins was not demonstrated.

While infection of mouse stem cells and expression of therpeuticaly relevant proteins has been demonstrated, there are still problems with this technique. Engraftment of infected cells is variable from mouse to mouse, even in animals receiving marrow cells from the same pool. In general, only a few infected stem cells engraft recipient animals, as shown by the small number of different virus integration sites in hematopoietic tissues from reconstituted animals. In addition, much of the repopulating ability of marrow is lost during the infection procedure, especially if the cells are selected for the presence of the vector before transplantation. These phenomena can be explained both by a loss of stem cells during infection and by inefficient stem cell infection. While these losses are acceptable in the inbred mouse model, where donor animals are not limited in number, they may prove a considerable obstacle to gene therapy in humans.

Attempts have been made to demonstrate stem cell infection and long-term gene expression in large outbred animals as a prelude to the application of these techniques to humans. Gene transfer into stem cells of such animals is more difficult to achieve because of the larger scale of the procedure and the fact that marrow is only available from the recipient individual, as would be true in human applications. In studies using monkeys and dogs, short-term expression of transferred genes was demonstrated, but long-term expression was not found.[25,26] However, in these studies 5-fluorouracil or other ablative agents were not used before marrow harvest, and infected cells were not preselected for the presence of the vector before transplantation. Thus, the results are not directly comparable with those in mice in which these techniques were used.

In utero transfer of the *neo* gene into fetal sheep has been accomplished[27] by removing circulating cells 7 weeks before birth, infecting these cells in vitro, and returning the infected cells to the fetus. Persistence of the infected cells after birth was demonstrated by detection of *neo* sequences in blood, bone marrow, spleen, and thymus samples from some animals 1 week after birth, detection of neomycin phosphotransferase in bone marrow of one animal 6 weeks after birth, and by the presence of G418-resistant hematopoietic colony-forming cells in marrow for more than 2 years after birth. However, the same infection procedure was not successful with marrow cells from adult sheep instead of fetal cells. This difference is most likely due to the high replication rate of fetal hematopoietic cells, which would make them more susceptible to infection by the retroviral vector.

The difficulty with infection of stem cells is in contrast to the successful infection of more differentiated hematopoietic progenitor cells from large animals, including dogs,[28,29] monkeys,[25] sheep,[27,30] and humans.[31-35] Infection rates range from a few percent to almost 40% depending on the vector used and infection conditions. Thus, retroviral vectors can be used to infect hematopoietic cells from species other than mice, including humans, suggesting that there are no fundamental reasons for the lack of success in infecting stem cells from large animals.

Given the difficulty of showing long-term gene expression in the hematopoietic system of large adult animals, new approaches have been pursued to improve gene tansfer rates. Evidence exists that marrow kept in long-term bone marrow cultures can be used to reconstitute humans and other animals[36,37] and methods to infect cells efficiently in long-

term culture are being developed for murine,[38,39] canine,[40] and human[34,41] hematopoietic cells. Techniques for purification of hematopoietic stem cells may also lead to improvements in stem cell infection rates. Methods have been developed for purifying early human hematopoietic cells[42] and for purifying early simian hematopoietic cells that are capable of engraftment in lethally irradiated baboons.[43] High rates of infection of purified pluripotential stem cells have been shown recently in the mouse.[44] The advantages of working with purified stem cells include the practical advantage of needing to infect fewer cells and the possibility that culture conditions, including growth factors, can be more precisely controlled to improve stem cell infection and survival. Purified hematopoietic growth factors improve stem cell infection rates,[7] and the use of these or other as yet unidentified factors may improve infection rates. Very high titer viruses generated by vector amplification during co-cultivation of different packaging cell lines also has been shown to improve infection rates in hematopoietic cells in mice and monkeys.[45] However, the low infection rate observed in monkeys (1% vector-positive circulating cells), the limited time of animal observation (up to 3 months), and the presence of helper virus in the vector preparations suggest that further improvements are necessary.

GENE TRANSFER INTO SKIN CELLS

Skin fibroblasts are potential targets for gene therapy because they can be easily explanted and grown in culture, and have been shown to persist after transplantation in a rat model system.[46] In an attempt to treat humans suffering from mucopolysaccharidoses, unmodified normal human skin fibroblasts have been transplanted subdermally into patients to provide a source of missing enzyme. Although transient metabolic improvements were observed in some studies,[47] fibroblast transplantation did not produce prolonged biochemical or clinical improvements.[48] Lack of prolonged improvement could be due to graft rejection, since the persistence of the transplanted fibroblasts was not established, or to insufficient levels of the required enzymes from the number of fibroblasts that were transplanted. Thus, the significance of these experiments to gene therapy using fibroblasts is not clear.

It has been tempting to assume that the immortal fibroblast cell lines familiar to all mammalian cell culturists provide a suitable model for gene therapy involving normal skin fibroblasts. However, there are important differences. Normal skin fibroblasts have a limited lifespan in culture[49] and suffer continuous changes in culture that may affect their ability to engraft after transplantation. Thus, gene transfer techniques should be efficient to minimize growth in culture, and cell cloning steps are probably not feasible. It is also important to avoid the use of immortalized cells that spontaneously arise during the culture of skin fibroblasts, especially with mouse cells, as these immortalized cells may behave differently than normal fibroblasts. Furthermore, gene transfer techniques that work well in continuous cell lines may not work well in normal fibroblasts. For example, normal fibroblasts are much more difficult to transfect than continuous cell lines by using calcium phosphate precipitation techniques. In addition, the control of gene expression from a given transcriptional unit may be quite different in normal fibroblasts compared with continuous cell lines[50]; thus, adequate gene expression in continuous cell lines may not predict the situation in normal fibroblasts.

Retroviral vectors provide a very efficient means to transfer genes into normal human fibroblasts, with efficiencies of over 50% in fetal, newborn, or adult skin fibroblasts.[51] By using retrovirus-mediated gene transfer, human fibroblasts have been shown to make biologically active ADA,[51] glucocerebrosidase,[52] purine nucleoside phosphorylase,[53] low-density lipoprotein receptors,[54] and factor IX.[50] In the case of factor IX, vector-infected fibroblasts produced up to 3 $\mu g/10^6$ cells/d. Thus, skin fibroblasts can be engineered to make a number of proteins, including those that require post-translational modification and secretion for activity. An example of the latter is factor IX, which must undergo glycosylation and gamma-carboxylation on glutamyl residues to have activity.

Collagen matrices containing genetically modified fibroblasts that secrete human factor IX have been implanted under the skin of animals.[50,55] Alternatively, the cells have been grown on collagen beads and injected intraperitoneally. Up to 190 ng/mL factor IX was detected in the plasma of some animals for short periods after transplantation.[50] Normal levels of factor IX in humans are about 5μg/mL, but 500 ng/mL would alleviate most of the symptoms of severe hemophilia B. Thus, the levels obtained approach therapeutic utility. However, expression was observed for no longer than a month in these animals. Antibodies against human factor IX were detected in some animals; thus, loss of expression could be due to an immune response. Alternatively, the transplanted cells may not have survived well, may have stopped synthesizing factor IX, or may eventually reside in a compartment that does not allow movement of factor IX into the bloodstream.

Skin keratinocytes may also provide suitable targets for gene therapy. Keratinocytes can be easily obtained and grown in culture to form large sheets that can be used for transplantation, and have been used to replace damaged skin in the treatment of burn patients.[56] Although the epithelium is not vascularized, and thus proteins made by keratinocytes must move through the epidermis and cross the basement membrane separating the epidermis from the dermis, it has recently been shown that a protein as large as apolipoprotein E (90 Kd) can reach the circulation after secretion by transplanted keratinocytes.[57] Thus, keratinocytes might be used for protein synthesis or for metabolic functions involving low molecular weight compounds.

Efficient gene transfer into keratinocytes has been accomplished by co-cultivation of the cells with virus-producing fibroblasts. Keratinocytes are normally grown on a feeder layer of fibroblasts, and the virus-producing cells can provide this function during infection. Transfer efficiencies of up to 80% have been reported for gene transfer into canine keratinocytes.[58] Keratinocytes are also capable of secreting biologically-active proteins after gene transfer, as shown for cultured human keratinocytes infected with a retroviral vector carrying the human growth hormone gene.[59] After

subdermal transplantation of such cells into athymic mice, growth hormone was detected in the implant for up to a week after transplantation. No circulating human growth hormone was detected in the recipient animals, although this may be due to the limited sensitivity of the assay. In other studies, canine keratinocytes carrying a retroviral vector that expressed the *neo* gene were shown to persist for at least 120 days after subdermal transplantation in dogs.[58] Keratinocytes cultured from such grafts still expressed the *neo* gene, suggesting that vector-encoded genes remain active in keratinocytes for long periods after transplantation.

GENE TRANSFER INTO HEPATOCYTES

Retroviral vectors have been used to transfer genes into primary cultures of mouse,[60,61] rat,[62-64] and rabbit[65] hepatocytes. Infection efficiencies of 25% have been reported.[63,64] Primary cultures of hepatocytes cannot currently be expanded in culture, and such cells can only divide a few times. Thus, it is important to transfer genes into these cells with high efficiency to obtain suitable numbers of cells for transplantation.

Methods for reimplantation of hepatocytes directly into the liver have not been developed, but hepatocytes have been implanted into intraperitoneal or subdermal sites to measure their function in vivo. Studies with unmodified normal hepatocytes have shown that hepatocytes attached to dextran beads will persist in the peritoneal cavity for 2 months, and will function to correct partially the defects in albuminemic Nagase rats or hyperbilirubinemic Gunn rats for up to a month after implantation.[66,67] Normal hepatocytes will also persist and function in organoid structures formed in the peritoneal cavity by implantation of polytetrafluoroethylene fiber supports coated with heparin-binding growth factor-1, which rapidly become vascularized and provide a support for implanted hepatocytes.[68,69] By using this technique, hepatocytes from normal rats have been shown to persist and reduce serum bilirubin levels in hyperbilirubinemic Gunn rats for at least 4 months. These results suggest that genetically modified hepatocytes may function appropriately in ectopic sites. Indeed, hepatocytes infected with a virus carrying the *neo* gene and grown on porous supports persisted and continued to make neomycin phosphotransferase after implantation under the skin or intraperitoneally.[64]

In addition to gene transfer into hepatocytes in vitro by using retroviral vectors, methods have been developed for direct gene transfer into hepatocytes in vivo.[70-74] One method relies on Sendai virus-mediated fusion of vesicles containing DNA mixed with proteins found in the cell nucleus (to facilitate DNA movement to the nucleus).[71,72] After injection of these vesicles into the portal veins of adult rats, DNA was detected in livers of the animals for about a week, but decreased rapidly thereafter. Protein expression was also detected for approximately a week, and the use of nuclear proteins improved expression in comparison with albumin, which was used as a control. Another method involves targeting of DNA to the liver by using DNA complexed with asialoglycoprotein.[73] The DNA complexes were shown to target specifically to the liver after injection of tail veins, and protein expression from a transferred transcriptional unit was detected 1 day after injection. Thus, both of these methods result in short-term gene expression in the liver. More recent studies have shown that partial hepatectomy before DNA delivery extends the expression time to longer than 2 weeks.[74] Whether these systems will be of practical use remains to be established.

GENE TRANSFER INTO ENDOTHELIAL CELLS

One virtue of vascular endothelial cells as targets for gene therapy is their direct access to the circulation. Thus, endothelial cells could be used for metabolic or protein secretion functions. Rabbit endothelial cells have been shown to make several proteins, and can secrete rat growth hormone at up to 1 $\mu g/10^6$ cells/d.[75]

Two model systems have been used to demonstrate persistence of transplanted endothelial cells in animals. Both models used retroviral vectors containing the bacterial gene encoding β-galactosidase, which is easily detectable by enzymatic histochemical procedures that result in blue staining of cells. In one model,[76] canine endothelial cells were shown to be infectable at greater than 50% efficiency by a retroviral vector carrying β-galactosidase. Infected canine endothelial cells were seeded on prosthetic Dacron vascular grafts and the grafts were surgically implanted in dogs. Analysis of grafts removed 5 weeks after implantation showed that the modified cells had persisted and were still making the bacterial β-galactosidase. In the other model,[77] infected porcine endothelial cells were seeded directly into denuded arteries of pigs, and persistent β-galactosidase expression was observed in arteries removed at 2 to 4 weeks post-implantation. Thus, vector-infected endothelial cells can be transplanted and will continue to express vector-encoded proteins for at least a month.

While these studies are encouraging, a potential problem with gene therapy aimed at endothelial cells is the limited number of endothelial cells present in large vessels. Since the layer of endothelial cells that lines the vessels is only one cell thick, the potential number of target cells is limited. If these techniques can be extended to endothelial cells in the periphery, a much larger number of cells will be available as targets. However, such considerations do not preclude use of endothelial cells to alter conditions locally, such as the use of endothelial cells to secrete anti-clotting factors to inhibit local thrombosis.[78]

GENE TRANSFER INTO MUSCLE CELLS

Recent experiments have shown that it is possible to transfer genes directly into mouse muscle in vivo by direct injection of DNA into skeletal muscle.[79] Injection of a plasmid containing the *Escherichia coli* β-galactosidase gene driven by the Rous sarcoma virus promoter resulted in β-galactosidase expression in individual myotubes in the area of the injection. Injection of a similar plasmid containing the luciferase gene resulted in expression of luciferase for 2 months at relatively constant levels, suggesting that expression may persist for longer periods. The injected DNA appeared to persist in an unintegrated extrachromosomal state. If expression after plasmid injection does persist long-term, this method will provide an exciting alternative to

the more complex methods for gene transfer that are currently used. However, this technique does not appear to work as well in tissues other than muscle.[79]

GENE TRANSFER INTO LYMPHOCYTES

The first experiment involving gene transfer into human patients involved gene transfer into T lymphocytes by using a retroviral vector. Tumor infiltrating lymphocytes (TIL) were grown from tumors of patients suffering from metastatic melanoma, infected with a retroviral vector in vitro, expanded, and returned to patients in an attempt to destroy cancerous cells. In this case, the cells were infected to mark the cells so that their fate could be determined in vivo, and gene expression was not required for the success of the experiment. The significant findings of the experiment were that infected TIL persisted in the patients, particularly at tumor sites, and there were no adverse effects from the procedure.[80] These results show the feasibility of retrovirus-mediated gene transfer in humans. It is likely that experiments involving modification of TIL to express proteins that assist in the destruction of tumor masses, such as tumor necrosis factor, will be proposed in the near future. These approaches are a logical extension of the TIL marking experiments and might lead to true gene therapy approaches for cancer treatment.

Recently a proposal has been submitted that involves the use of geneticaly altered T cells to treat ADA deficiency. T cells from ADA-deficient patients would be infected with a retroviral vector encoding ADA and returned to the patient. In contrast to the TIL cell marking experiments, continued gene expression would be necessary in this case. Although lymphocytes have only a finite life span in vivo, this treatment could be repeated for continued therapy. This protocol has been approved by internal National Institutes of Health review committees, and recently was approved by the Human Gene Therapy Subcommittee of the Recombinant DNA Advisory Committee on June 1, 1990. Major issues that were addressed were the adequacy of available treatments for ADA deficiency, particularly the direct injection of polyethylene glycol-modified ADA enzyme (PEG-ADA),[81] and whether the potential benefits of gene therapy for these patients outweigh the risks, given that there are alternative therapies.

CONCLUSIONS

Much progress has been made in the development of gene transfer techniques suitable for use in gene therapy and in the evaluation of a variety of somatic cells types as potential targets for such therapy. However, difficulties remain with all of these approaches to gene therapy. While levels of ADA expression obtained in mouse hematopoietic cells appear to be sufficient for treatment of human ADA deficiency, there are problems with application of the bone marrow modification techniques to large outbred animals. Although skin fibroblasts can provide potentially useful levels of circulating factor IX in rodents, expression is short-lived. While hepatocytes can be efficiently infected in vitro and have a potentially large synthetic and metabolic capacity in vivo, methods for their transplantation into the intact liver or into ectopic sites must be improved. Direct transfer of DNA into liver or muscle in vivo appears promising, but further development will be necessary to evaluate its potential. Thus, progress is needed to understand factors that control expression from genes transferred into somatic cells, to develop suitable methods for tissue transplantation after gene transfer in vitro, and to extend the experiments using direct gene transfer. Given the exciting initial results in marking cells with transferred genes in humans, it is reasonable to expect rapid progress in the application of these bold new techniques to disease treatment.

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Transfer and Expression of Cloned Genes Using Retroviral Vectors

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INTRODUCTION

It has been a little more than 10 years since the genetic engineering revolution began. Initially, "genetic engineering" applied only to manipulation of genes in bacteria, being a technology which enables the isolation and large scale production of specific DNA molecules from any source, whether or not they encode a gene. If properly engineered, such genetic manipulations can be used for the large scale production of specific gene products in bacteria and other simple organisms.

Genetic engineering of higher eucaryotic or mammalian cells requires the introduction and expression of genes in these cells. One method for transferring genes into mammalian cells is DNA transfection in which DNA is introduced into cells in culture as part of a coprecipitate with calcium phosphate or dextran sulfate (26). A successful result is a viable cell containing one to many copies of the new gene which continuously expresses the new genetic information. Unfortunately, DNA transfection has its limitations. Most significantly, it is a very inefficient means of transferring genes into mammalian cells. At best only one in a thousand cells (more typically, one cell in a million) will incorporate the newly transferred gene. Additionally, not all cultured cell lines are susceptible to this method of gene transfer. Thus, a central issue in genetic engineering as applied to mammalian cells is gene transfer: How to deliver a gene into a large fraction of any given cell population. A second important issue is the question of expression — once delivered, how to ensure the proper expression of the gene in the recipient cell.

In the past several years, a new gene transfer technology has emerged which appears to be superior to the DNA transfection and other previous techniques and which may offer a new approach to the therapy of human genetic diseases (1). This new technology is known as retroviral-mediated gene transfer, i.e., the use of retroviruses to deliver genes into cells.

In order to explain how retroviruses can be utilized for gene transfer, it is important to describe some of the salient features of their replication (For more detailed information on retroviruses, see Ref. 25.). Retroviruses are RNA viruses, that is, the viral genes are encoded in an RNA molecule rather than in a DNA molecule. When the virus

penetrates a cell, the viral RNA is first converted to DNA, the DNA enters the nucleus and integrates randomly into a chromosome, becoming indistinguishable from any other cellular gene (see Fig. 1). It is from this integrated form, the *provirus*, that the viral genes are expressed. Progeny virus are formed which leave the cell by budding from the cell membrane. It is important to point out that the integration of the viral genome into the cell's chromosome is an essential part of its replication. With a few exceptions, the presence of the viral genome in the infected cell, the expression of its genes, and the formation of progeny virus have no apparent effect on the viability of the infected cell. Thus, retroviruses have features that make them particularly suitable for gene transfer. By replacing the viral genes with the gene of interest and using the efficient viral integration process, it is now possible to transfer a gene into the infected cell as if it were a viral gene.

How does this work? The viral genome, encoded in an RNA molecule in the virion and in a double stranded DNA in the infected cell, contains two types of information which can be classified as cis and trans functions. The trans functions are the viral proteins such as the polymerase and the envelope glycoprotein. The cis functions are the various signals scattered throughout the viral genome, such as the promoter and enhancer sequences required for initiation of RNA transcription. Other examples of cis functions are the various sequences which direct the integration of the viral genome into the chromosome of the infected cell as well as the encapsidation signal (Ψ) required for virus packaging. To construct a virus that can be used as a vector, the cis functions must be retained while the trans functions can be replaced by the gene of interest. What is thereby created is a replication defective retrovirus missing its own genes. The trans functions which were removed can be supplied by another virus (called a *helper virus*) in the same cell or by a second defective virus that still contains the trans functions missing in the vector. This mechanism for obtaining viability by combining the activities of two defective components (commonly used in somatic cell hybridization) is called *complementation*. Thus, the retroviral vector carrying the gene of interest can be assembled into a virion, exit from the cell, infect a target cell, and, through the cis functions retained in the vector, the foreign gene is transferred into a chromosome of the cell as if it were a viral gene.

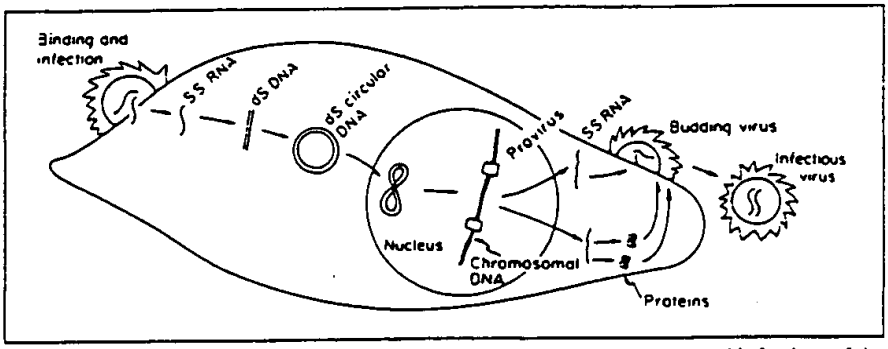

Figure 1. The retroviral life cycle. After adsorption and uptake (i.e., binding and infection) of the viral genome, the single-stranded (SS) RNA is converted to double-stranded (DS) DNA by reverse transcriptase. After uptake into the nucleus, the DNA integrates randomly into a chromosome to form the provirus. The provirus serves as a template both for mRNAs to yield viral proteins and for new, full length genomic RNA. The genomic RNA and viral proteins combine and, by budding, release a new infectious virus to repeat the cycle. (Reprinted with permission from A. Bernstein *et al*, in Genetic Engineering: Principles and Methods, p. 235, Plenum, NY, 1985.)

In the laboratory, this process takes place in two steps as shown in Fig. 2. First, a preparation of retrovirus is generated (by, for example, transfection of a packaging cell line, see below) that contains the foreign gene (this is, therefore, a *recombinant virus*); second, the gene is delivered to the target cell by infection of the target cells with the recombinant virus. Recombinant DNA techniques allow the manipulation of DNA sequences, but not RNA sequences. Therefore, the initial procedure is to combine the portions of the retroviral DNA carrying the cis functions, with the DNA fragment carrying the foreign gene (and removing in the process the viral trans functions). How is this vector DNA converted into a corresponding vector RNA and encapsidated into a virus? To do this, the vector DNA is introduced by the standard (however inefficient) DNA transfection procedures into a specially designed cell line called a *packaging cell* (18, 22). The packaging cell line harbors a virus that is defective in the cis function that is required to be present for the viral RNA to be able to encapsidate into a virion. All its trans functions are normal, however, so that they will complement those functions missing from the incoming vector DNA. It is then possible to identify and isolate the rare cells that have taken up the vector DNA by use of a *selectable gene* present in the vector. For example, cells infected with a vector containing a Neo^R gene are resistant to toxic levels of the neomycin-like antibiotic, G418.

The vector DNA not only is transcribed into RNA which is translated within the cell, but also it is transcribed into viral genomic RNA which is encapsidated into a retroviral virion, and secreted into the medium. The recombinant virus carrying the foreign gene can now infect a target cell and then integrate into its genome, carrying the foreign gene with it. Since the viral RNA carrying the trans functions cannot encapsidate, the virions carry *only* vector RNA. These particles can infect only once since the viral RNA they contain have no trans function genes. The particles are a one-time-only delivery system.

At present, it is now possible to insert a gene into a retroviral vector, obtain recombinant virus, and then infect target cells and express the foreign gene from the cells' chromosomes. What has proven more difficult is to make this process *efficient*. An efficient gene transfer system is desirable for its usefulness to basic research, but is an absolute prerequisite for application to human therapy (1). Indeed, a large ef-

fort is currently underway in several laboratories to improve this technology. Two parameters define an efficient retroviral gene transfer system. First is the capacity to infect a large proportion of the target cells, a property dependent at least in part on the ability to generate a high concentration (or *titer*) of recombinant virus. Second is the capacity to have the gene expressed properly.

RETROVIRAL VECTORS

One of the key components of this technology is the nature of the retroviral vector itself. It is the vector that will determine both the titer of recombinant virus obtained and the ability of the transduced gene to be expressed. The development of an all-purpose superefficient retroviral vector has proved elusive, arising from the fact that we do not understand many of the details of the structure and biology of this group of viruses. Ongoing research on the basic biology of these viruses is, therefore, vital for the development of improved vector systems.

Three main strategies of retroviral vector construction are shown in Figures 3-5. A short description and discussion of their properties will follow. Note that these vectors carry not one but two genes. One gene is the gene of interest and the second one is a selectable gene. This second gene is not absolutely essential to include in the vector but its presence greatly facilitates their use. A selectable gene, as its name implies, enables the identification and isolation of cells harboring the retroviral vector as was discussed above. In some cases, the gene of interest is a selectable gene and therefore only one gene is incorporated (4, 12, 20, 21).

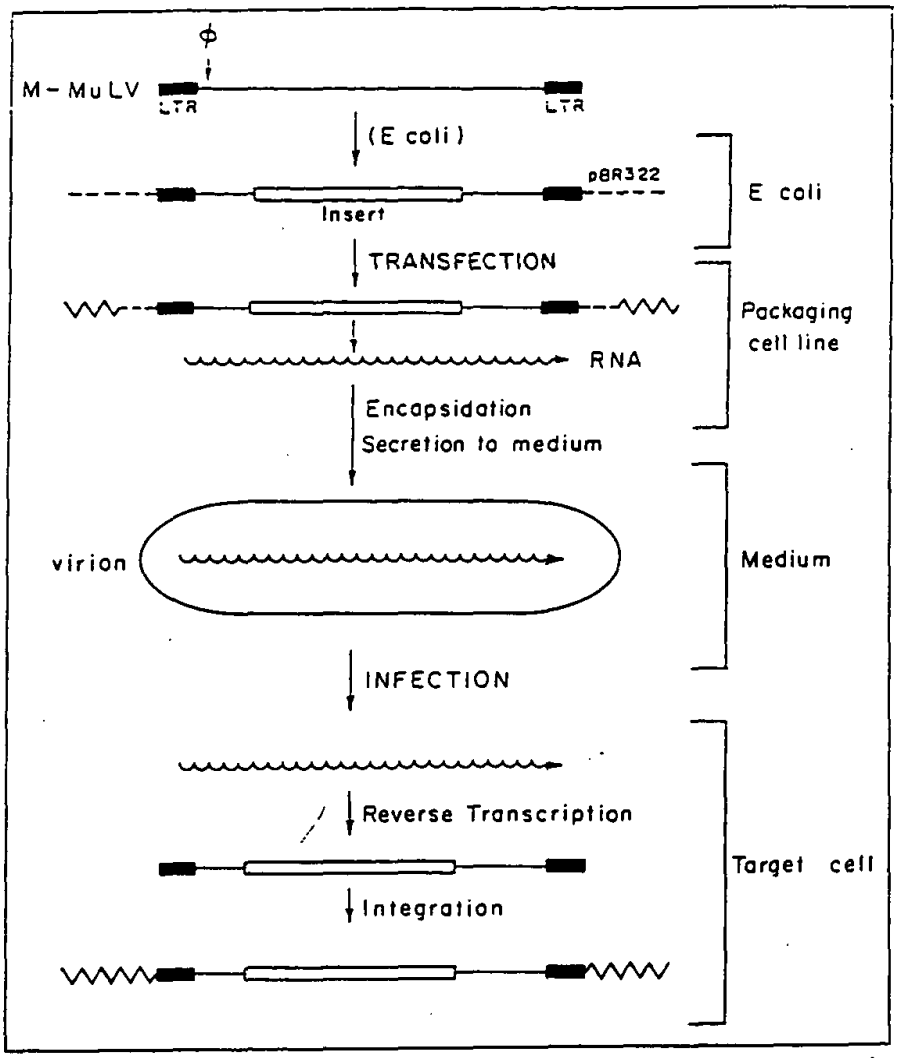

Figure 2. Generation of retroviral vectors. A native retrovirus (M-MuLV: Moloney-Murine Leukemia Virus) is genetically engineered such that its structural genes are replaced by an exogenous gene (5). This construct is cloned into *E. coli* and the DNA is then introduced by conventional transfection (step 1-see text) into a packaging cell line. These cells provide the structural proteins in trans to permit both packaging of the vector and budding of vector-containing viral particles (infectious virus) into the medium. Target cells are infected (step 2) with these viral particles, where the natural cycle of reverse transcription and integration occurs. The stably integrated vector provirus then generates its gene product(s).

Double Expression (DE) Vectors

The structure of a prototype double expression DE vector is shown in Fig. 3. In retroviruses, most of the important cis functions are present at the ends of the viral genome and are maintained in the corresponding vectors. The termini of retroviruses are redundant and are called *long terminal repeats*, or, in short, LTRs. They are represented in Fig. 3 and henceforth by black boxes.

As shown in Fig. 3, the MuLV viral genes are expressed from two mRNA forms. The gag and pol genes are expressed from an unspliced RNA species which is colinear with the viral genome. The env gene is expressed from a spliced RNA form generated from the unspliced RNA species by the removal of the long intron. In the cytoplasm of the infected cells, similar amounts of both mRNA species are present and, therefore, the retroviral genome must regulate the efficiency with which the intron is removed. DE vectors as shown in Fig. 3 contain two foreign genes, one replacing in effect the gag/pol genes (which is expressed from the unspliced RNA form) and a second gene replacing the viral env gene (which is expressed from the spliced RNA form). The distinguishing feature of this type of vector is that it provides not only the cis functions for the transmission of the foreign genes into the target cells but also provides the cis functions for their

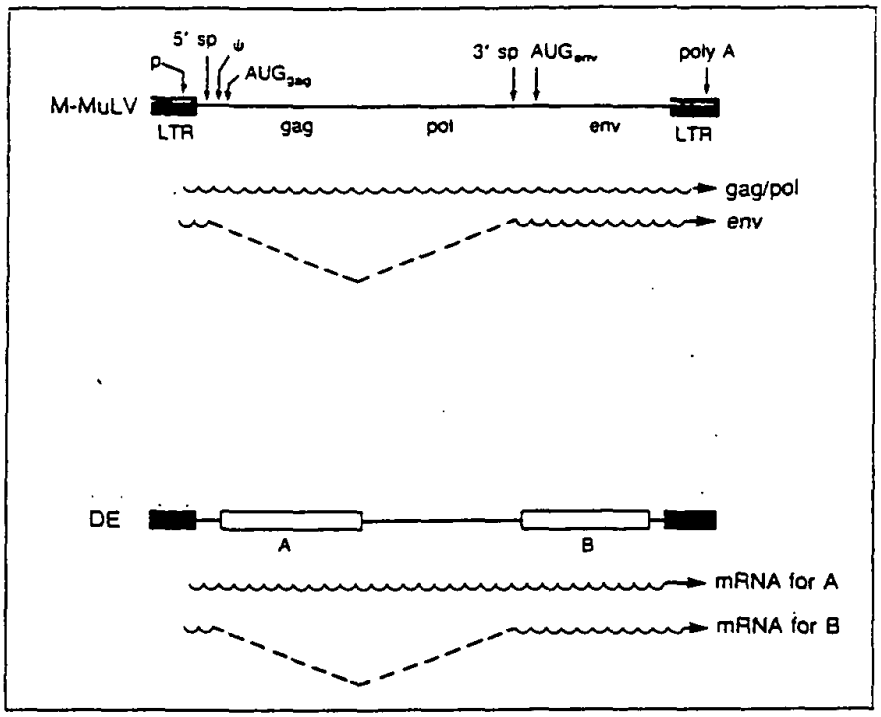

Figure 3. DE vectors. M-MuLV (Moloney-Murine Leukemia Virus) generates two types of transcripts (wavy lines): Unspliced (gag/pol) and spliced (env) RNA, both under the regulation of the LTRs (black boxes). In a DE vector the gag/pol and env genes are replaced with exogenous genes A and B. Then, the normal splicing mechanisms are used to generate mRNAs for genes A and B, with expression of both regulated by the 5 ' LTR.

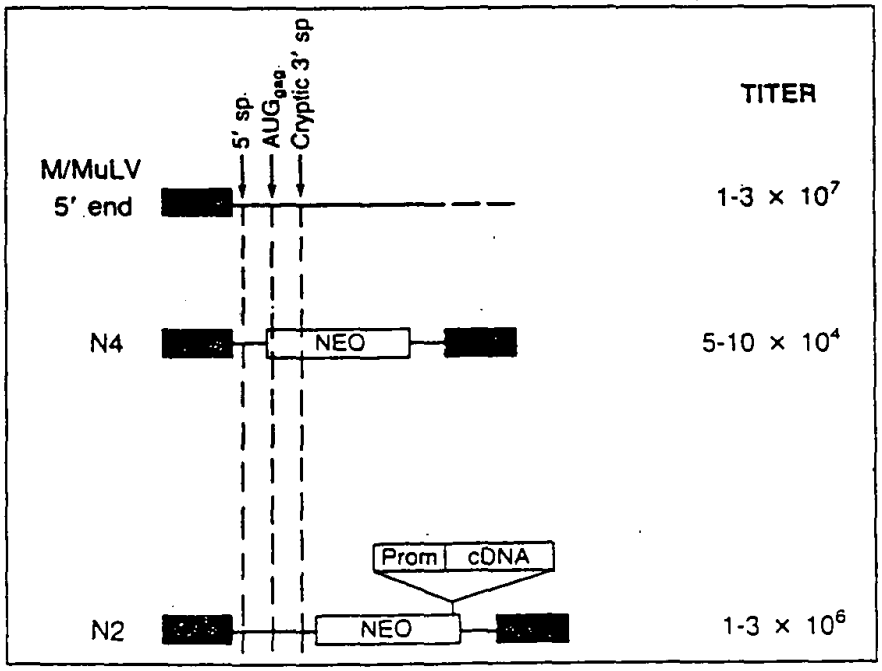

Figure 4. VIP vectors. VIP vectors rely only on the very 5 ' portion of the retroviral genome for their function. A selectable gene product (e.g., Neo^R) is generated from a transcript regulated by the retroviral 5 ' LTR. A second gene of interest is inserted distally, under the regulation of its own promoter, generating a separate, unique mRNA. Titers (viral particles/ml) are listed at the right, showing wild type titers with those of two VIP vectors, N4 and N2.

expression, i.e., the enhancer and promoter present in the 5 ' LTR, the polyadenylation signal in the 3 ' LTR, and the intron splice sites encoded internally. Such a DE type vector was first reported by Cepko, et al. (2), and used successfully in several studies (3, 7, 17, 24, 27, 28).

There are two major drawbacks in using DE vectors. First, the expression of the two genes introduced into DE vectors is dependent on the efficient formation of the two viral RNA species as shown in Fig. 3, which, in turn, is dependent on a properly regulated splicing process. In the construction of the DE vectors described by Cepko, et al. (2), it was assumed that it is the viral splice junction sequences which regulate the splicing process. However, there is now mounting experimental evidence that this is not the case; rather, sequences within the viral intron play an essential role (10, 11, 19) in modulating the levels of spliced and unspliced RNA forms present in the cytoplasm. The second major drawback of the DE type of vector is that the transduced gene is expressed from the retroviral promoter and, therefore, its usefulness will be limited to target cells where the viral promoter is active and to those experiments where this is the promoter that one wants to study.

Vectors with Internal Promoters (VIP)

VIP vectors were designed to circumvent these limitations. As shown in Fig. 4, in these vectors the selectable gene is directly linked to the left end of the viral DNA and is therefore expressed from the viral promoter. The gene of interest, however, is fused to another DNA fragment containing a promoter which is responsible for its expression. This promoter DNA fragment can be derived from any gene and, therefore, these vectors possess the flexibility to express the transduced gene in a manner most appropriate for a particular target cell (6, 23).

Figure 4 also shows the structure of our primary VIP vector, N2. This vector yields very high titers of virus (1-3 x 10^6 cfu/ml) compared to other vectors (of the DE or VIP design) and efficiently expresses the selectable gene, in this case the bacterial Neo^R (neomycin

resistance) gene. Interestingly, if one examines the structure of the vector, it should not have worked at all. It was originally made as part of a study to determine the length of the packaging sequence (D. Armentano, Ph.D. Thesis, Princeton University, 1986). In contrast to other VIP vectors such as N4, in N2 418 bp of the viral gag coding sequences (including the start code word) has been retained, but the Neo^R gene is out of frame and therefore one might have assumed that it would not be expressed. However, it appears that it is this gag coding sequence which is responsible for the higher levels of virus generated from N2 vectors. It turns out that this DNA has unexpectedly introduced a cryptic 3 ' splice site just upstream from the Neo^R gene which generates a spliced RNA form that serves as the mRNA for the Neo^R gene (Armentano, et al., submitted). Evidence is accumulating from several laboratories that the N2 vector and its derivatives are exceptionally useful retroviral vectors, especially for the transfer of genes into suspension grown lymphoid cells (13) and bone marrow progenitors of several mammalian species (5, 9, 15, 16). (More on this topic below.)

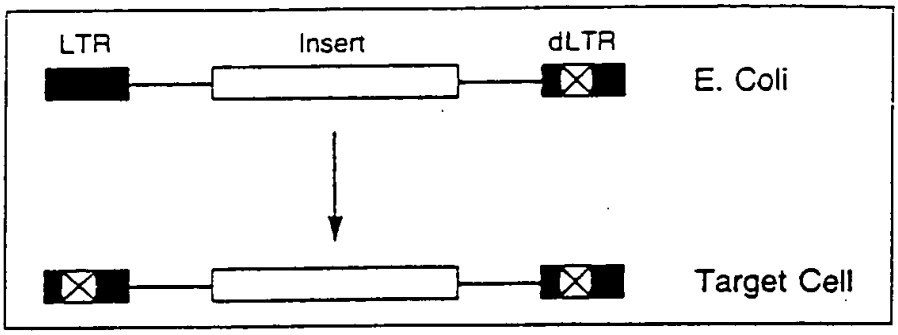

Figure 5. The deleted LTR trick. An initial vector is constructed in a plasmid such that the promoter/enhancer elements are deleted from the 3 ' LTR (dLTR). Upon transfection into a packaging cell line, this structure is maintained. When the resulting vector is introduced into target cells, the 3 ' LTR is used as a template for both LTRs during the process of reverse transcription. Therefore the provirus in the target cell has both LTR promoter/enhancer sequences deleted, leaving the vector genes reliant on internal promoters for expression.

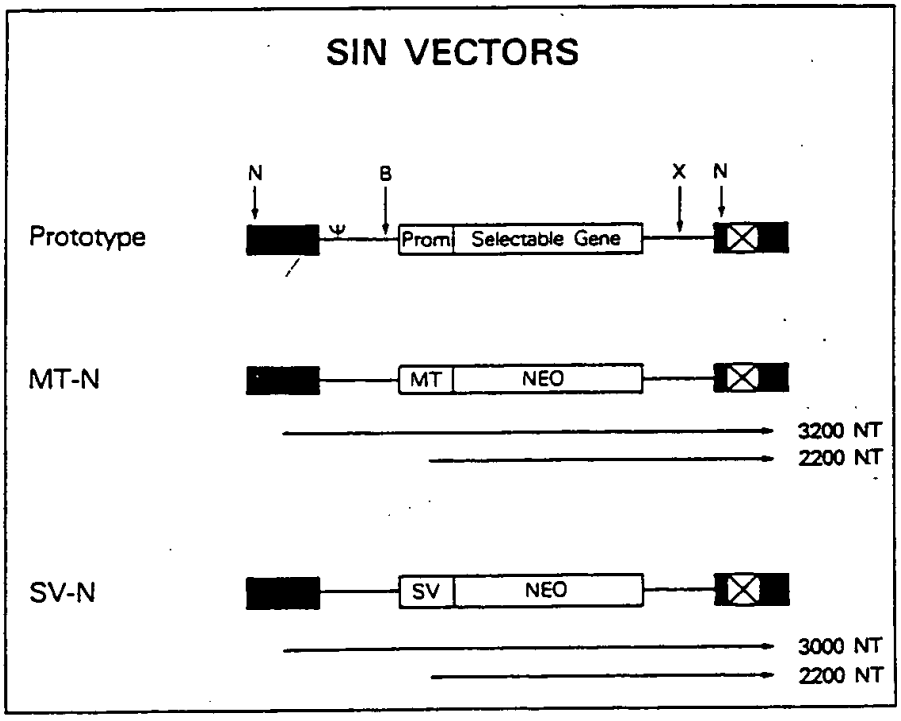

Figure 6. SIN vectors. The cloned structure of a prototype SIN vector is shown at the top. MT-N and SV-N are specific examples of such vectors, where the neomycin resistance gene (Neo^R) is under the regulation of a metallothionein (MT) or SV40 (SV) promoter. After infection, the 5 ' LTR would be disabled as described in Figure 5. Therefore, only the shorter transcripts produced with the internal promoter would be generated.

Self-Inactivating (SIN) Vectors

SIN vectors are the latest addition to retroviral vectors (29). The LTRs at the two ends of the retroviral genome contain an element called an enhancer which not only can affect the expression of the vector's gene but, when integrated into a cell's chromosome, can also activate adjacent oncogenes (a process called insertional activation) (8). SIN vectors are designed to eliminate these two effects by "self-inactivating". During the process of reverse transcription and integration, a portion of the virus DNA which includes the enhancer and promoter sequences becomes deleted. As a result, the proviral DNA in the infected cells becomes transcriptionally inactive, thus producing two consequences: The uninhibited expression of the foreign gene and the reduction of insertional activation.

How this self-inactivation works is shown in Figure 5. SIN vectors contain a 299 bp deletion in the 3 ' LTR (dLTR). This deletion encompasses the promoter and enhancer sequences which control the accurate and efficient transcription of the viral genome. Only the sequences in the 5 ' LTR are necessary for the generation of viral RNA. Therefore, their removal from the 3 ' LTR, as shown in Fig. 5, does not affect viral functions. A consequence of the replication of retroviruses (25) is that a region of the 3 ' LTR (which encompasses this deletion) is the template for the synthesis of that region in both the 5 ' and 3 ' LTRs. Since the viral enhancer and promoter are absent from

the template, they will be absent from both LTRs in the target cells.

The structure of a prototype SIN vector is shown at the top of Figure 6. The selectable gene is expressed from an internal promoter (cloned into the vector along with the gene) rather than from the viral LTR as with the VIP vectors discussed in the previous section. Since, upon infection of the target cells, the promoter activity in the viral LTR is inactivated, the expression of the selectable gene in the target cells is controlled by its own internal promoter. SV-N and MT-N are two examples of SIN vectors shown in the lower portion of Fig. 6; the early SV40 (SV) or mouse metallothionein I (MT) promoters are used respectively to drive the expression of the selectable gene coding for neomycin resistance (Neo^R). The gene of interest can be inserted into SIN vectors either in front of or behind the selectable marker, utilizing the presence of unique restriction sites (BamHI and XhoI, respectively, Fig. 6).

Although it has been demonstrated that SIN vectors indeed self-activate in the target cell (29), the titer of virus generated from this type of vector are disappointingly low (~10^4 cfu/ml), probably not sufficient for use in human therapy. It is hoped that appropriate modifications, together with advances in the understanding of the retroviral genome, will increase the performance of SIN vectors.

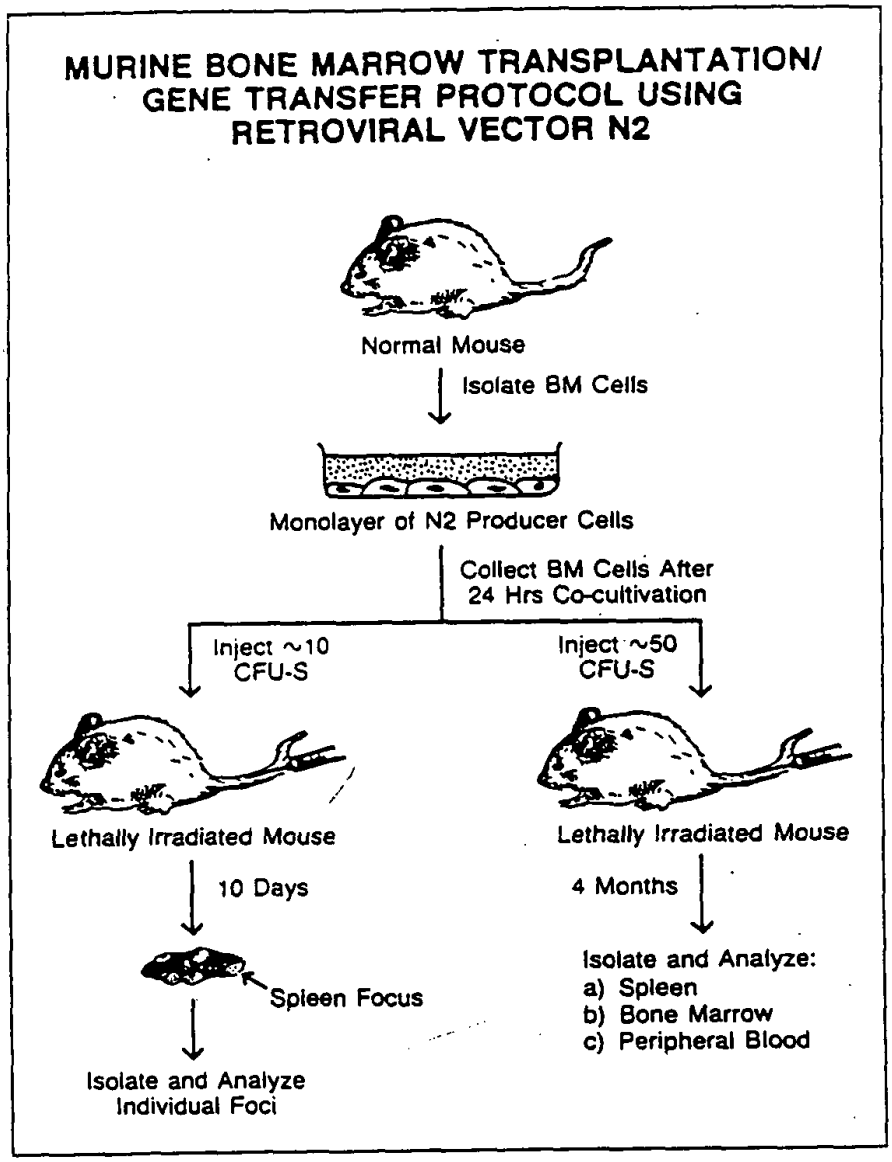

Figure 7. Outline of the murine bone marrow transplantation/gene transfer protocol. Bone marrow (BM) cells are isolated from the femur, exposed to the vector by co-cultivation with vector producer cells, and then introduced into lethally irradiated recipient mice. CFU-S are progenitor cells whose lineage is assayable by colonies (or foci) formed in the spleen. The number of spleen foci are proportional to the total number of stem cells injected.

EXPRESSION

Retroviral vectors are much more efficient for the insertion of DNA sequences than other presently available means of gene transfer. They are valuable not only for cells growing attached to a substratum, but also for cells grown in suspension culture. This latter quality has given geneticists access to a great many types of cells previously refractory to genetic engineering.

If hematopoietic stem cells could be infected with a vector, the potential exists for new genetic material to become a permanent part of the blood tissue's genetic complement. Stem cells are most abundant within the bone marrow. Yet even there they are present only as a tiny fraction (less than one cell in a thousand) of the total nucleated cells. It is only by means of retroviral vectors, with their combined characteristics of very high efficiency and applicability to cells in suspension, that genetic manipulation of the totipotent stem cells of the hematopoietic system has become practical.

Several laboratories have published protocols by which the hematopoietic cells of mice have had new genes introduced with retroviral vectors *ex vivo*, with subsequent reintroduction of the treated cells into the bone marrow *in vivo*. The basic outline of such a bone marrow transplantation/gene transfer protocol is very simple (Fig. 7). Marrow cells are obtained from a donor and incubated with a monolayer of vector-generating producer cells (27). Since marrow cells, unlike the producer cells, do not attach to the culture dish, they

Table 1. Summary of Spleen Focus Analysis From Bone Marrow Infections

Cell Line	Hours of Culti-vation	Initial Titer	−IL3 Spleens Studied	−IL3 Total Foci	−IL3 Average Foci per Spleen	−IL3 Foci Ana-lyzed	−IL3 # DNA Postive	−IL3 Percent DNA Positive Foci	+IL3 Spleens Studied	+IL3 Total Foci	+IL3 Average Foci per Spleen	+IL3 Foci Ana-lyzed	+IL3 # DNA Positive	+IL3 Percent DNA Positive Foci
F-5B	24 hr.	$1.2x10^6$	2	10	5.0	9	7	78	8	94	11.8	49	42	86
	48 hr.	$2.0x10^6$	1	8	8.0	8	6	75	8	71	8.9	18	14	78

are easily recovered after co-cultivation. The hematopoietic cells are then introduced into a recipient animal by intravenous injection. Space in the hematopoietic system to receive the vector-treated marrow must be made, usually by lethally irradiating the recipient.

Using this protocol, highly efficient introduction of exogenous genes into hematopoietic progenitor cells have been achieved. Experiments, such as those presented in Table 1, have shown that greater than 85% of spleen focus stem cells (so called CFU-S) can be infected with the N2 vector (5). Viability of the stem cells does not appear to be impaired, and the vector has been shown to integrate in its intact form. In some cases, relatively low efficiencies have been improved by pre-selection of infected marrow prior to introduction into a recipient mouse (4, 15). Advantage is taken of the presence of a selectable gene in the vector to enrich the total population of marrow for cells containing the vector. Incubation in high concentrations of the selective agent for one or two days between infection and transplantation has improved the recovery of infected cells between three- and ten-fold (4, 15).

Not surprisingly, the titer of the vector is of great importance in the successful transfer of exogenous genes into hematopoietic progenitor cells. In general, titers of at least 10^4 cfu/ml seem to be required (Fig. 8). Although vector instability appeared to be a problem in early efforts at gene transfer into bone marrow (12), recent experiments with helper-free vectors show this to be a reduced concern (4, 5, 15). Minimizing the viral sequences retained in the vector reduces the likelihood of recombinatorial disruption of the vector.

Expression of transferred genes in tissue culture cells is very efficient. At present, N2 is the only vector that has been consistently shown to exhibit efficient expression (of the Neo^R gene) in the mature hematopoietic cells of long-term reconstituted mice (5, 15). Drug resistant colony forming progenitor cells and enzymatically active protein can be obtained from mice several months after gene transfer (Fig. 9 -right panel). Thus, these initial mouse studies have borne out the potential of retroviral vectors for the high efficiency transfer of intact, functioning vectors stably into bone marrow cells.

We have added an SV40-promoted human adenosine deaminase (ADA) gene to the N2 vector as shown in Fig. 10. This VIP vector, called SAX (for SV40, human ADA, inserted into the XhoI site), has been used to introduce the ADA gene into the hematopoietic cells of several species other than mouse (Table 2). Lymphocyte lines from both

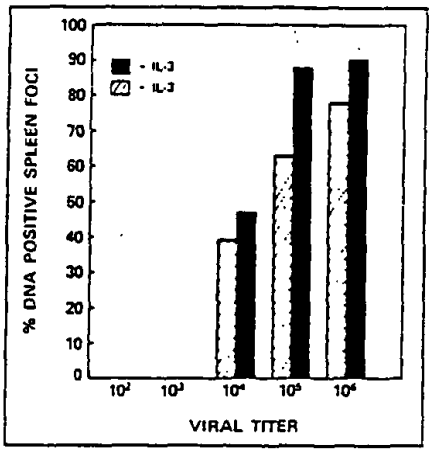

Figure 8. Effect of titer on infectivity of murine CFU-S. Bone marrow was treated with vectors of the titers shown. Titer is expressed by the number of drug resistant colony forming units (cfu) per ml assayed *in vitro* on a tissue culture cell line (usually 3T3 cells, a murine fibroblast cell line). A titer of 10^4 cfu/ml seems critical for successful infection. Interleukin-3 (IL-3), which is a growth stimulating factor, may improve infection slightly by stimulation of the bone marrow.

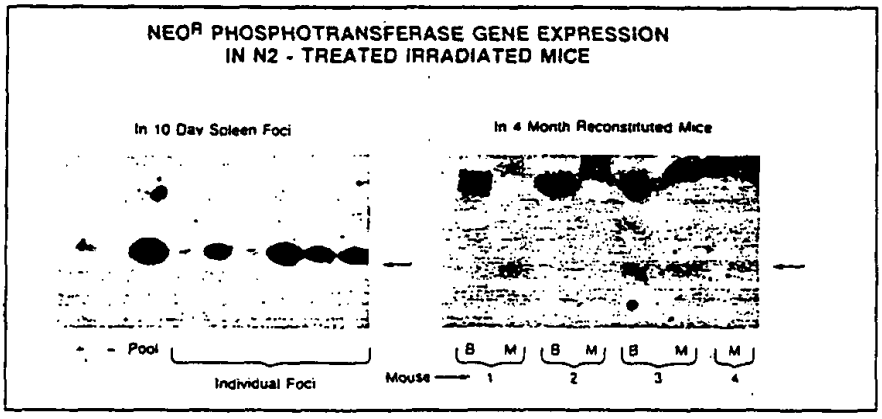

Figure 9. Neo^R phosphotransferase gene expression in N2-treated irradiated mice. Bone marrow cells were infected with the N2 vector as shown in Fig. 7. In the left panel, individual spleen foci were isolated and assayed for the presence of the Neo^R-gene-coded phosphotransferase (see Ref. 5). In this case, all six individual foci had detectable levels of enzyme activity, although amounts varied. +, positive tissue culture control; -, uninfected spleen foci cells; Pool, activity of 8 pooled foci from one spleen. In the right panel, four mice were analyzed four months after receiving sufficient numbers of treated bone marrow cells to reconstitute their entire hematopoietic system (see Fig. 7). In three of four mice (#1, 3, and 4), low levels of enzyme activity were still detectable in the bone marrow (M). In one of these three mice (#3), activity was also detected in the blood (B).

Table 2. Successful Transfer and Expression Using N2 (or N2-like) Retroviral Vectors into Hematopoietic Precursors of Various Species

SPECIES	VECTOR	ASSAY	REF.
Mouse	N2	CFU-S, mature cells	(5)
	N2	CFU-GM, mature cells	(14)
	Neoμ	GEMM, CFU-GM, BFU-E	(4)
Dog	N2	CFU-GM	(16); a
Human	N2	GEMM, CFU-GM, BFU-E	(9) b
	N2	GEMM, CFU-GM	b
	N2	CFU-GM, BFU-E	c
	SAX	ADA$^+$ T cells	(13)
Monkey	N2, SAX	CFU-GM, mature cells	d
Sheep	N2	GEMM, CFU-GM, BFU-E, CFU-E	e

Neoμ is a vector similar to N2, as described in (4). SAX is a VIP vector derived from N2 containing the human adenosine deaminase cDNA regulated by an SV40 promoter (13).

a) Lothrop, C., Eglitis, M. et al., unpublished observations.

b) Dupree, J., personal communication; Bernstein, A., personal communication; *Humphries, R.K., personal communication.*

c) Eglitis, M. et al., unpublished observations.

d) Kantoff, P., O'Reilly, R., Nienhuis, A. et al., unpublished observations.

e) Zanjani, E., Kantoff, P., Flake, A. et al., submitted for publication.

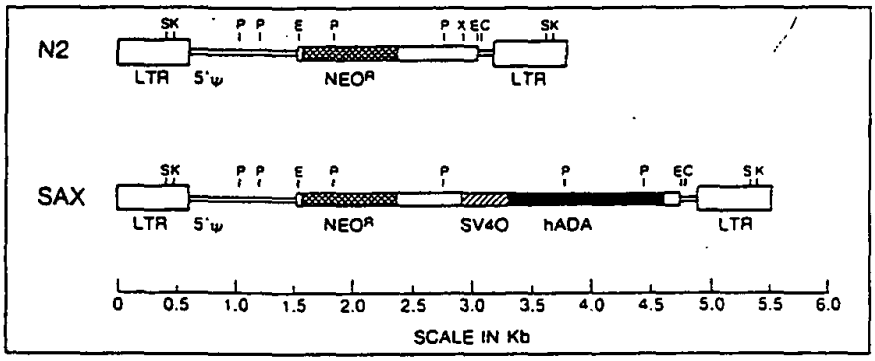

Figure 10. Map of SAX vector. SAX was made (13) by inserting an SV40-promoted human ADA cDNA into the previously described (5) parental vector N2. The following regions are indicated: 0-1.5 and 4.7-5.5 kb, Moloney murine leukemia virus sequences; 1.5-2.8 and 4.6-4.7 kb, neomycin-resistance gene (Neo^R) from Tn5 transposon (the hatched area is the coding sequence); 2.9-3.3 kb, Kpn I-Hind III fragment of the SV40 early promoter; 3.3-4.6 kb, human ADA cDNA (hADA, black box); LTR, viral long terminal repeat; Ψ, viral packaging signal. Restriction sites: S, Sac I; P, Pst I; E, EcoRI; C, Cla I.

monkeys and humans have been successfully infected, with significant expression of the Neo^R and ADA genes (13, 14). Bone marrow of humans, dogs, sheep and monkeys have been infected and assayed *in vitro*. Productive transfer of exogenous genes has been achieved with each species tested, although, in general, at efficiencies lower than those found for mice. Successful transfer and expression of genes into bone marrow *in vivo* has also been obtained in both sheep and non-human primates (14). Extensive studies are now underway to increase the overall efficiency of gene transfer in these and other large animals.

PERSPECTIVES

Retroviral vectors have proven to be an efficient means of gene transfer. They will undoubtedly be applied to a wide range of genetic studies of eukaryotic cells. Even more exciting is the possibility of their use for the treatment of human genetic disease. However, many aspects of the structure and function of retroviruses must still be elucidated to optimize them as vectors. Particularly in the use of retroviral vectors for gene transfer into large animals, problems remain in the efficiency of infection of pluripotent stem cells and in the long-term stability of expressed genes. If these problems can be solved, then the next few decades could well see retroviral vectors at the heart of a revolution in the medical treatment of genetic disease.□

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Prospects for Human Gene Therapy

W. French Anderson

Gene therapy, the insertion into an organism of a normal gene which then corrects a genetic defect, has been carried out in fruit flies (*1*) (*Drosophila melanogaster*) and mice (*2*). How soon gene therapy might be available for the treatment of human genetic diseases and what criteria should be used in determining when clinical trials should begin are issues examined in this article. Several investigators are now preparing protocols for clinical trials of gene therapy in seriously ill patients (*3*). Since most of these protocols will be based on the use of retroviral vectors as a delivery system, these structures will be emphasized. It may well be, however, that one of the other delivery systems described below, or a new one not yet developed, will be the procedure of choice in the future.

Summary. Procedures have now been developed for inserting functional genes into the bone marrow of mice. The most effective delivery system at present uses retroviral-based vectors to transfer a gene into murine bone marrow cells in culture. The genetically altered bone marrow is then implanted into recipient animals. These somatic cell gene therapy techniques are becoming increasingly efficient. Their future application in humans should result in at least partial correction of a number of genetic disorders. However, the safety of the procedures must still be established by further animal studies before human clinical trials would be ethical.

Gene Therapy in Lower Species

The most elegant system thus far demonstrating successful gene therapy is the work in *Drosophila* (*1*). The transposable genetic element, the P factor, has been used to transfer a normal gene coding for the enzyme that produces the wild-type red eye color in *Drosophila* embryos which have a genetically defective gene. The result is that the treated flies acquire normal eye color. Similar transfer experiments under way use other genes. Despite considerable searching, transposon-like elements have not been identified in vertebrates. Retroviruses, however, are structurally and functionally similar in many ways to the mobile genetic elements found in lower organisms, and retroviral vectors have now been used to transfer functioning genes into mouse bone marrow cells.

The first genetic "cure" reported in a mammal (*2*) was in a strain of mice, called *little*. These have a mutation that results in reduced serum levels of growth hormone, and the mice are therefore dwarfs. The equivalent human disease is pituitary dwarfism. Hammer *et al.* (*2*) succeeded in inserting a rat growth hormone gene into the cells of these mice in such a way that the gene is expressed at a high level. The deficiency in growth hormone was corrected, and the animals grew rapidly, but the gene was not controlled appropriately, and gigantism resulted—namely, a mouse one-and-a-half times as large as a normal animal. A major research effort is focusing on how to correctly regulate transferred genes.

Gene Therapy in Humans

Human disease candidates for gene therapy. Pituitary dwarfism in humans is not a reasonable initial candidate. Genes making hormones that circulate in the bloodstream are probably not appropriate for early attempts at gene therapy in humans. First, the normal feedback controls in DNA that regulate the expression of hormone genes in the body are not now known. Therefore, physiologically correct levels of hormone production would probably not be possible. Second, it would be easier and safer to use recombinant DNA manufacturing techniques to produce sufficiently large quantities of hormone so that the active polypeptide itself could be given to the patient. Hormone levels could then be titrated precisely.

At first, clinical investigators thought that the human genetic diseases most likely to be the initial ones successfully treated by gene therapy would be the hemoglobin abnormalities (specifically, β-thalassemia) because these disorders are the most obvious ones carried by blood cells, and bone marrow is the easiest tissue to manipulate in vitro (*4*). Regulation of globin synthesis, however, is unusually complicated. Not only are the embryonic, fetal, and adult globin chains carefully regulated during development, but also the α- and β-globin-like chains are always maintained in a 1 to 1 ratio despite the fact that the α- and β-globin loci are on different chromosomes. To understand the regulatory signals that control such a complicated system and to develop means for obtaining controlled expression of an exogenous β-globin gene will take considerably more research effort. The recent development of a mouse model for β-thalassemia should aid these investigations (*5*).

Gene therapy should be beneficial primarily for the replacement of a defective or missing enzyme or protein that must function inside the cell that makes it, or of a deficient circulating protein whose level does not need to be exactly regulated (for example, factor VIII). Early attempts at gene therapy will almost certainly be done with genes for enzymes that have a simple "always-on" type of regulation. Three genes are the initial prime candidates: hypoxanthine-guanine phosphoribosyl transferase (HPRT), the absence of which results in Lesch-Nyhan disease; purine nucleoside phosphorylase (PNP), the absence of which results in a severe immunodeficiency disease; and adenosine deaminase (ADA), the absence of which results in severe combined immunodeficiency disease. For all three, the clinical syndrome is profoundly debilitating. The defect in each is found in the patient's bone marrow (although the severe central nervous system manifestations of Lesch-Nyhan disease are due to absence of HPRT in brain cells and probably cannot be corrected with current techniques). In all three there is no, or minimal, detectable enzyme in marrow cells from patients homozygous (or hemizygous) for the defect, and the production of a small fraction of the normal enzyme level should

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be beneficial. Furthermore, a mild overproduction of enzyme should not be harmful to the cell. In addition, in all three the gene has been cloned and a complementary DNA is available.

Since combined immunodeficiency due to a defect in the ADA gene in T lymphocytes can be corrected by infusion of normal bone marrow cells from a histocompatible donor, selective replication of the normal T cells appears to take place (*6*). This observation offers hope that defective bone marrow can be removed from a patient, the normal ADA gene inserted into a number of cells through gene therapy, and the treated marrow reimplanted into the patient where it may have a selective growth advantage. There is also evidence that marrow cells containing HPRT ($HPRT^+$) may have a selective advantage (in both mice and humans) over cells that do not ($HPRT^-$) (*7*). If selective growth occurs, no ablation of the patient's own marrow would be necessary. If, however, corrected stem cells have no growth advantage over endogenous ones, then partial or complete marrow destruction (either by irradiation or by other means) may be required in order to allow the corrected marrow cells an environment favorable for expansion.

Ethics. The ethics of gene therapy in humans has been discussed for many years (*8*) and is being widely debated at present (*9*). Essentially all observers have stated that they believe that it would be ethical to insert genetic material into a human being for the sole purpose of medically correcting a severe genetic defect in that patient—that is, somatic cell gene therapy. Attempts to correct germ cells (that is, to permit the new gene to be passed on to the patient's children) or to enhance or improve a "normal" person by gene manipulation do not have societal acceptance at this time (*9*). However, somatic cell gene therapy for a patient suffering a serious genetic disorder would be ethically acceptable if carried out under the same strict criteria that cover other new and experimental medical procedures (*10*). The techniques that are now being developed for human application are for somatic cell, not germ line, gene therapy.

The question examined here is: What criteria should be used in evaluating gene therapy protocols? Three general requirements, first presented in 1980 (*10*), are that it should be shown in animal studies that (i) the new gene can be put into the correct target cells and will remain there long enough to be effective; (ii) the new gene will be expressed in the cell at an appropriate level; and (iii) the new gene will not harm the cell or, by extension, the animal. These three requisites, summarized as delivery, expression, and safety, will each be examined in turn.

Delivery

At present, the only human tissues that can be used for gene transfer are bone marrow and skin cells. No other cells can be extracted from the body, grown in culture to allow manipulation, and then successfully reimplanted into the patient from whom the tissue was taken. In the future, as more is learned on how to package the injected DNA and to make it tissue- or even cell type–specific, the intravenous route would be the simplest and most desirable. Attempting to give a foreign gene by injection directly into the bloodstream is not advisable with our present state of knowledge, since the procedure would be enormously inefficient and there would be little control over the DNA's fate (*11*).

Studies are considerably more advanced with bone marrow than skin cells as a recipient tissue for gene transfer. Bone marrow consists of a heterogeneous population of cells, most of which are committed to differentiation into erythrocytes, lymphocytes, megakaryocytes, and so on. Only a small proportion (0.1 to 0.5 percent) of nucleated bone marrow cells are stem cells (that is, cells that have not yet differentiated into specific cell types and which divide as needed to maintain the marrow population). In gene therapy, stem cells would be the primary target. Because they are low in number and are not recognizable, a delivery system for transferring a gene into stem cells must be efficient.

Techniques for transferring cloned genes into cells can be grouped in four categories: (i) viral, both RNA viruses (or retroviruses) and DNA viruses (for example, SV40, adenovirus, and bovine papilloma); (ii) chemical, such as calcium phosphate–mediated DNA uptake; (iii) fusion, that is, fusion of DNA-loaded membranous vesicles, such as liposomes, red blood cell ghosts, or protoplasts, to cells; and (iv) physical, that is, microinjection or electroporation. Each technique is valuable for certain types of experiments, but none can yet be used to insert a gene into a specific chromosomal site in a target cell. Fusion techniques are the least well characterized and will not be discussed. As noted, retroviral-based vectors appear to be the most promising approach at present for use in humans.

Viral Techniques

RNA viruses (retroviruses). There are a number of advantages of vectors derived from retroviruses as a gene delivery system. First, up to 100 percent of cells can be infected and can express the integrated viral (and exogenous) genes; this is in contrast to chemical methods where, although most cells take in the DNA, as shown by positive assays after 48 hours, only one cell in 10^3 to 10^7 stably expresses the exogenous gene. Second, as many cells as desired can be infected simultaneously; 10^6 to 10^7 is a convenient number for a simple protocol. Third, under appropriate conditions the DNA can integrate as a single copy at a single, albeit random, site, whereas the chemical and physical techniques often result in the insertion of multiple copies of the transferred gene, all linked head-to-tail in tandem repeats. Fourth, although integration is random with respect to the host genome, it is precise with respect to the viral genome—that is, the structure of the integrated DNA is known. Fifth, the infection and long-term harboring of the retroviral vector usually does not harm cells. Finally, a wide and controllable host range is available. A number of retroviral vector systems have been developed. Here we concentrate on vectors based on Maloney murine leukemia virus (MoMLV).

1) Life cycle and structure. The details of the life cycle of retroviruses have been reviewed recently (*12*). In brief, the retrovirus, composed of an RNA-protein core and a glycoprotein envelope, enters a cell where the RNA acts as a template for the reverse transcription of the genetic information into a double strand of DNA. This DNA can precisely integrate as a single copy, called a provirus, at a random location in the genome of the host.

Although much has been learned about the regulatory features of retroviruses, uncertainties remain. Those features of the proviral structure that are thought to be necessary for transcription and transmission of the viral genome are (see Fig. 1): a long terminal repeat (LTR) sequence on each end, containing regulatory signals for initiating and terminating transcription, sequences required for reverse transcription and others for proviral integration; short sequences (called here, for short, r^- and r^+) immediately adjacent to each LTR and necessary for reverse transcription; the packaging sequence called ψ in MoMLV, necessary for the viral RNA to be packaged into an infectious viral particle; and the donor (D) and acceptor (A) splice sites.

Retroviral RNA is synthesized from the proviral DNA by the host cell's own RNA polymerase. A portion of this RNA is used in the cell's translational machinery to synthesize the viral proteins that go into the final viral particles along with the genomic RNA. These viral particles bud off from the cell and can then infect other cells.

From experimental studies as well as the existence of a number of naturally occurring defective viruses, it is known that almost all of the regions coding for viral proteins (*gag*, *pol*, and *env* in Fig. 1) can be deleted and some or all of these sequences replaced with other DNA. Once the viral genes are deleted, the retroviral vector becomes defective. In order to obtain infectious viral particles, a cell harboring a defective provirus must be infected with a "helper" virus, which carries all the viral functions needed—that is, the genes for *gag*, *pol*, and *env*.

2) Use as gene delivery system. The proviral DNA for the desired retrovirus [commonly either MoMLV or murine sarcoma virus (MSV)] is isolated and inserted into a convenient plasmid. The viral genes can then be replaced with the exogenous genes of choice by standard recombinant DNA techniques. This construct is used to transfect tissue culture cells (for example, NIH 3T3 cells) by a convenient gene transfer procedure (for example, calcium phosphate). After infecting the cells with a helper virus (such as intact MoMLV), infectious viral particles, possessing both the retroviral vector and the helper virus, bud off from the cells into the surrounding medium. This particle-containing supernatant is collected and used to infect bone marrow cells in culture or, more simply, freshly extracted bone marrow is incubated directly with the cells budding the viral particles. The marrow cells are removed and injected intravenously into a mouse whose bone marrow has been killed by x-rays (lethally irradiated). The animal is then studied to determine if the transferred marrow cells express the desired gene from the vector.

3) Successful gene transfer into adult mice. Joyner *et al.* (*13*) have successfully used this procedure to transfer a functional gene for neomycin resistance (*neo*^{r}) into mouse hematopoietic progenitor cells by use of a MoMLV retroviral vector. The presence and expression of this gene in granulocytic progenitor cells rendered these cells resistant to the neomycin-like antibiotic G418 as determined by in vitro colony assays. Treated cells were injected into lethally irradiated mice; Southern blot analysis and colony assays showed that the *neo*^{r} gene is present and functional in the spleens of the recipient animals (*13*).

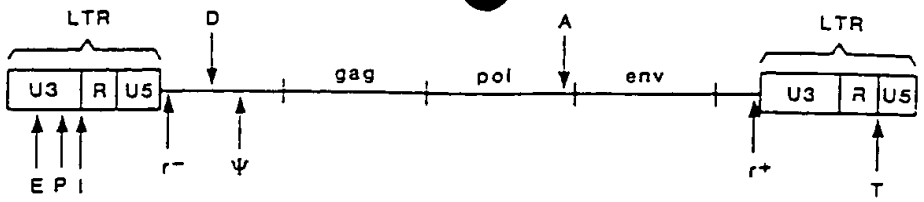

Fig. 1. Simplified structure of MoMLV retroviral provirus DNA. Abbreviations: E, enhancer; P, promoter; I, initiation (Cap) site for viral RNA synthesis; r$^-$, replication initiation site for minus DNA strand (transfer RNA binding site); D, donor splice site; ψ, packaging sequence; A, the major acceptor splice site; r$^+$, replication initiation site for plus DNA strand (purine-rich site); T, terminal [poly(A) addition] site for viral RNA synthesis; LTR, long terminal repeat; U3, R, and U5 are portions of the LTR; *gag*, group-specific (that is, viral core) antigens: p15, p12, p30, and p10; *pol*, RNA-dependent DNA polymerase (reverse transcriptase); and *env*, envelope proteins: gp70, p15E, and R. (Not drawn to scale)

An improvement on this procedure would be to treat bone marrow cells with a retroviral particle that could deliver the vector but which would not itself produce a spreading infection. Mann *et al.* (*14*) have developed a technique for accomplishing this goal. The regulatory signal ψ (Fig. 1) contains a sequence, the exact size and structure of which are not yet known (*15*), that must be present in the viral RNA for it to be packaged into a viral particle. A helper virus was constructed with this sequence deleted (ψ$^-$) by making use of convenient restriction endonuclease sites (Bal I and Pst I) flanking the ψ sequence in MoMLV. The ψ$^-$ helper is able to produce all the viral proteins required to make a particle, but the particle does not package its own RNA. Since the retroviral vector has a ψ sequence, it is packaged. Consequently, the particle can just infect once; it is only a delivery system for the vector, not an infectious agent.

In order to use the ψ$^-$ helper virus conveniently, a line of NIH 3T3 cells was established with the helper proviral DNA permanently integrated (*14*); ψ$^-$ helper viral RNA is produced constitutively. The transfection of this cell line (called ψ-2) with the retroviral vector DNA results 48 hours later in a supernatant that contains viral particles with only the vector.

Williams *et al.* (*16*) have used the ψ-2 cell line to place a functioning *neo*^{r} gene into the hematopoietic cells of adult mice. Freshly extracted murine bone marrow was layered onto ψ-2 cells producing a retroviral vector called MSV DHFR-NEO, which contains genes for dihydrofolate reductase (DHFR) and *neo*^{r} in an MSV backbone. The marrow and ψ-2 cells were incubated for 48 hours under standard incubation conditions; similar results were obtained when bone marrow cells with the ψ-2 MSV DHFR-NEO cell layer were incubated for 6 days under Dexter-type conditions (*17*). The viral particles that budded off into the supernatant contained the MSV DHFR-NEO vector but, to the extent that could be determined, no ψ$^-$ helper viral RNA. Ten days after lethally irradiated mice were injected with the treated bone marrow cells, analysis of the regenerating spleens showed that the mice carried the MSV DHFR-NEO proviral DNA in their hematopoietic cells. Individual spleen colonies, each arising from a single stem cell, were generated by injecting an estimated one to ten stem cells into another group of lethally irradiated mice. Cells from individual colonies were able to produce spleen colonies in a secondary group of lethally irradiated animals. These mice also were shown to carry MSV DHFR-NEO DNA in their total spleen DNA and, in each case, to have the same integration site restriction pattern as the colony from the primary mouse. These data show that the delivery system is effective, at least for mouse bone marrow cells. Preliminary evidence indicates that the *neo*^{r} gene is expressed (*16*).

Southern blot analysis of total spleen DNA with a number of restriction enzymes revealed in some cases a small number of proviral integration sites. This result suggests that only a few infected stem cells were proliferating to repopulate the irradiated spleen. Secondary transfers of individual colonies showed that only 7 of 48 colonies (15 percent) contained the *neo*^{r} gene. This is a lower limit since an occasional colony might have been formed from endogenous stem cells that survived the irradiation. These data suggest that the present bone marrow procedure might still be made more efficient as a delivery system.

A similar retroviral vector system based primarily on MoMLV has been developed by Verma and his co-workers (*18*). In their ψ$^-$ helper virus they substituted an amphotrophic (that is, wide host

range) *env* gene for the MoMLV *env* gene, which produces a particle coat with a narrow host range. This helper viral construct is called pSAM. Miller *et al.* (*19*) built a retroviral vector containing a full-length complementary DNA for the human enzyme HPRT. This vector, called pLPL, was cotransfected along with the ψ^- helper, pSAM, into HPRT$^-$ BALB/3T3 cells. One clone (c7cl) was obtained that produced high levels of viral particles containing the HPRT vector. Injection of these cells into lethally irradiated mice resulted in animals that continued to produce HPRT-vector particles for at least 6 months (*19*). The infectious particles resulted from the presence of low levels (<0.1 percent of the HPRT-vector virus) of packageable helper virus along with the injection of MoMLV as additional helper which led to multiple rounds of replication in the host. In addition, human HPRT enzyme was detected in spleen cells.

4) Shortcomings of retroviral delivery systems. The evidence indicates that retroviruses can be used as a reasonably efficient delivery system. A gene therapy procedure, however, also requires a reliable system. In most of the work reported to date, a number of cells are found to contain altered proviral DNA. The biggest problem appears to be that retroviruses have a strong propensity for deleting sequences during virus replication (*19a*). Many vectors have been ineffective because the foreign DNA is partially or totally removed from the construct or is rearranged. For example, Joyner and Bernstein (*20*) have used the Friend spleen focus-forming virus as a potential vector system for hematopoietic cells. Constructs containing a thymidine kinase (TK) gene in the *gag* region and an intact *env* gene (gp55) were used, along with MoMLV as helper, to obtain viral particles. The particles were injected into lethally irradiated mice and also layered onto rat TK$^-$ (LTA) cells. Southern blot analysis of the integrated proviral DNA in erythroleukemic spleens demonstrated vector constructs with intact gp55 genes but deleted TK sequences, whereas TK$^-$ LTA clones possessed intact TK genes but deleted or rearranged gp55 sequences. In other words, in no case could a provirus be found that still contained both the TK and gp55 genes. Even the successful MSV DHFR-NEO vector, which produces *neo*^{r} expression in mice, has lost a portion of its DHFR gene during production of the viral particles (*16*). Several approaches are being tried to circumvent this problem of instability (*19a*).

5) Properties still needed for an optimal delivery system. An ideal delivery system not only would be stable but also would be tissue-specific. When a genetic disorder is in the hematopoietic system, then the isolated bone marrow can be treated. But no other tissue, except skin cells, can be removed, treated, and replaced at present. Since many viruses are known to infect only specific tissues (that is, to bind to receptors that are present only on certain cell types), a retroviral particle containing a coat glycoprotein that recognizes only human hematopoietic stem cells would permit the retroviral vector to be given intravenously with little danger that cells other than those in the marrow would be infected. Such specificity could permit the liver and brain, for example, to be treated individually. In addition, the danger of inadvertently infecting germ cells could be eliminated. One problem, however, is that cell replication appears to be necessary for integration. It would not be possible to infect nondividing brain cells, for example, as far as we now know.

The optimal system not only would deliver the vector specifically into the cell type of choice but would also direct the vector to a predetermined chromosomal site. Specific insertion into a selected site of a chromosome by means of homologous recombination can be readily achieved in lower organisms but appears to be a formidable task in mammals, whether retroviral vectors or plasmid-based vectors are used. Present evidence suggests that homologous site-specific integration occurs at a very low level, when it occurs at all, in mammals (*21*).

DNA viruses. Viruses, such as SV40, with DNA as the nucleic acid in their core have been employed for several years as gene transfer vectors (*22*). A conditionally nonreplicating adenoviral vector has recently been developed that will efficiently infect animal and human cells (including hematopoietic cells) with the result that one or a few copies of the recombinant virus are integrated into the host cell's genome (*23*). Whether adenoviral vectors will be as efficient as retroviral vectors, or will offer other advantages as a gene transfer delivery system, remains to be determined. One subcategory of DNA viruses should be mentioned: bovine papilloma virus (BPV) (*24*). This viral DNA replicates extrachromosomally so that BPV-based vectors may prove to be useful for maintaining genes in cells in a nonintegrated manner. Transfection of hematopoietic cells with BPV-vectors has not yet been reported.

Chemical Techniques

The other procedure under active consideration for insertion of genes into human bone marrow cells is calcium phosphate–mediated DNA uptake. The original procedure of Graham and van der Eb (*25*) was modified by Wigler *et al.* (*26*) in order to insert into the genome of mammalian cells growing in culture a fragment of DNA carrying one or more genes. A number of genes have been used including the herpes simplex TK gene complementing TK$^-$ cells, the DHFR gene protecting against the drug methotrexate, and the *neo*^{r} gene protecting against the antibiotic G418.

Procedure. Transfection is carried out by pipetting a suspension of DNA, complexed into small precipitates with calcium phosphate, onto a monolayer of cells growing in a tissue culture dish (*26*). A number of techniques are used to increase the efficiency of transfection in different cell types: for example, diethylaminoethyl dextran can be employed instead of calcium phosphate or the cells can be shocked with glycerol after 2 hours of incubation (*27*). The efficiency of the process varies with the cell line. Under optimal conditions and very receptive cells (for example, mouse L cells), one cell in 10^2 to 10^3 can be obtained that has integrated and expressed the exogenous DNA. Because the usual efficiency is 10^{-5} to 10^{-7}, a procedure is required to detect the occasional transfected cell. In other words, a gene must be present that can protect the cell from a lethal selective agent that is added to the incubation medium or that complements a genetic defect (HPRT or TK, for example). The transfected cell will survive while all others are killed. Attempts to obtain transfected cells without selective pressure have generally been unsuccessful.

Transfection appears to work poorly in suspension cells, namely bone marrow cells. Efficiencies can only be estimated, but the value is probably one cell in 10^6 or 10^7. Using the powerful selection system offered by the mutant DHFR gene (isolated from 3T6-R400 cells) that provides exceptional resistance to methotrexate, Carr *et al.* (*28*) reported that the calcium phosphate transfer technique can be successfully employed to obtain mouse bone marrow cells that contain a functional exogenous DHFR gene. The permanently transfected cells can partially repopulate a lethally irradiated mouse. These results support the studies of Cline *et al.* (*29*) who reported successful transfer of a functional DHFR gene into the bone marrow of mice. However,

the presence of the DHFR gene has not been confirmed with DNA hybridization studies and, until such experiments are reported, the efficiency of the calcium phosphate procedure is uncertain.

Shortcomings of chemical techniques. If a chemical technique for gene transfer were used in a protocol designed for humans, the predicted results appear discouraging. Recovery from bone marrow of approximately 10^{10} nucleated cells (of which 10^7 to 10^8 are stem cells) can routinely be obtained from patients for marrow transplantation. Efficiency of 1 in 10^6 would mean that only 10 to 100 stem cells would be transfected. Reinsertion of these cells into the total stem cell pool of 10^8 to 10^9 cells would be very unlikely to have any noticeable effect on a patient's course unless there was an extraordinary selective advantage for the treated cells. Any human gene therapy protocol that uses chemical means for transfection would have to establish, therefore, that either a few transfected stem cells might have a detectable beneficial effect on the patient's course or that the investigator has improved substantially the efficiency of the procedure for human bone marrow cells.

Physical Techniques

Microinjection (*30*) and electroporation (*31*) are the two principal classes of physical techniques. Electroporation, a relatively new technique, is the transport of DNA directly across a cell membrane by means of an electric current. It has been used to transfer a variety of genes into a number of different cells including the immunoglobulin κ gene into B cells (*31*). Its potential for human gene therapy is uncertain.

Microinjection has been used for a number of years and has the advantage of high efficiency (up to one cell in five injected can be permanently transfected). However, the distinct disadvantage is that only one cell at a time can be injected. Transfection of a large number of hematopoietic stem cells is not feasible. Even if a stem cell could be recognized it would have to be fixed to a slide for injecting. The effect of attaching, injecting, and subsequent detaching is unknown. Microinjection of mouse erythroleukemia (MEL) cells is difficult, although possible (*32*), and these cells are much easier to manipulate in culture than are bone marrow cells.

Transfer of genes into mouse eggs. An area where microinjection has had spectacular success is in transferring genes into fertilized mouse eggs (*33*). Gordon *et al.* (*34*) first demonstrated that if plasmid DNA is microinjected into one of the two pronuclei of a recently fertilized mouse egg, and the ovum is then placed into the oviduct of a pseudopregnant female, the egg could develop into a normal mouse carrying the plasmid DNA in every cell of its body. Furthermore, the injected DNA can be transmitted to offspring in a normal Mendelian manner. Mice carrying an exogenous gene in their genome are called "transgenic."

Hammer *et al.* (*2*) used this technique to partially correct a mouse with a defect in its growth hormone production. By attaching a rat growth hormone gene to an active regulatory sequence (specifically, the promoter that normally directs the synthesis of metallothionein messenger RNA in mice), they obtained a recombinant DNA construct that actively produces growth hormone in the genetically defective mouse. Although the level of growth hormone production is inappropriately controlled—that is, influenced by signals that normally regulate metallothionein synthesis—these experiments do show that microinjection can be used as a delivery system that can put a gene into every cell of an animal's body.

Nonapplicability for humans. Should the technique of microinjecting a fertilized egg be employed for human gene therapy at the present time? The answer is no on three grounds: the procedure has a high failure rate, can produce a deleterious result, and would have limited usefulness. Microinjection has a high failure rate because the majority of eggs are damaged by the microinjection and transfer procedures so that they do not develop into live offspring. In one recent experiment involving microinjection of an immunoglobulin gene (*35*), 300 eggs were injected, 192 (64 percent) were judged sufficiently healthy to be transferred to surrogate mothers, only 11 (3.7 percent) proceeded to live birth and 6 (2 percent) carried the gene. These results are from a highly experienced laboratory in which thousands of identical eggs from the same hybrid cross of inbred mice have been injected over a number of years. The mice were chosen precisely because they gave the best results for gene transfer by microinjection. Experience with attempts to microinject growth hormone genes into livestock eggs have met with a number of major biological and technical problems (*36*). Successful gene transfer by microinjection of human eggs, without a long period of trial and error experimentation, is extremely unlikely.

Second, microinjection of eggs can produce deleterious results because there is no control over where the injected DNA will integrate in the genome. Lacy *et al.* (*37*) showed that the integration of an exogenous rabbit β-globin gene in transgenic mice could sometimes occur into a chromosomal location that results in expression of the β-globin gene in inappropriate tissue, namely, muscle or testes. There have been a number of cases reported where integration of microinjected DNA has resulted in a pathological condition (*38*). Although there is no control over where exogenous DNA will integrate in any gene transfer procedure, the damaging effect caused by a harmful insertion site could be great when it occurs in the egg but may be negligible when it occurs in one or a few of a large number of bone marrow cells.

Third is limited usefulness. Not only is it of questionable ethics to experiment on human eggs because of the expected losses, but even if "success" were obtained, it would be applicable primarily when both parents are homozygous for the defect. When the parents are both carriers, only one fertilized egg out of four would result in an affected child (*39*). Since a homozygous defect cannot yet be recognized in an ovum, and since the procedure itself carries such a high risk, it would be improper to attempt any manipulation in this situation. Furthermore, most of the very serious genetic disorders result in infertility (or death before reproductive age) in homozygous patients. Consequently, there would be little use for the procedure even if it were available. A different approach for human gene therapy is required.

Expression

The second criterion for evaluating a human gene therapy protocol is that there be appropriate expression of the new gene in the target cells: Even when a delivery system can transport an exogenous gene into the DNA of the correct cells of an organism, it has been a major problem to get the integrated DNA to function. A vast array of cloned genes have been introduced into a wide range of cells by the several gene transfer techniques discussed above. "Normal" expression of exogenous genes is the exception rather than the rule.

Active exogenous promoters in transgenic mice. Microinjection of fertilized eggs with exogenous DNA to obtain transgenic mice carrying an expressing gene has resulted in several spectacular successes, but also in a considerable number of unpublished failures. Thus far

only four genomic promoters have been reported to show significant activity: metallothionein (*2*), transferrin (*33*), immunoglobulin (*35*), and elastase (*40*). However, essentially any complementary DNA can be attached to an active promoter, such as metallothionein, and the coding sequence will usually be expressed in a transgenic mouse under the control of that promoter.

Why are most promoters inactive after microinjection into mouse oocytes? At least one promoter has been examined in this regard: mouse β^{maj} globin. The sequences are found to be heavily methylated in mouse tissues where they are inactive but relatively unmethylated in tissue culture cells where they are active (*41*). Therefore, the mouse zygote appears to respond to this foreign DNA by covering it with methyl groups which remain on the DNA throughout the lifetime of the animal. Attempts to decrease the methylation of the genomic DNA by treating adult mice carrying an exogenous β-globin promoter with the hypomethylating drug *5*-azacytidine have been essentially unsuccessful (*41*). The metallothionein promoter, however, even if methylated, can remain active (*42*). Why some promoters are inactivated by methylation, or other mechanisms, while others are not is not known.

Expression from retroviral vectors. If a retroviral vector is used for gene transfer, the transcriptional signals in the retrovirus's own LTR's can be used (Fig. 1). Expression of exogenous genes carried by retroviral vectors into bone marrow cells has been reported by three laboratories. The two studies in which a *neo*^{r} gene was expressed in mouse bone marrow were described above (*13*, *16*). The most extensive data, however, are from Willis *et al.* (*43*). A homozygous Lesch-Nyhan (LN) lymphoblast cell line was used to determine whether an HPRT$^-$ human hematopoietic cell could be corrected by a retroviral vector containing a functional HPRT gene. The LN cells have all the characteristics of a cell line totally defective in HPRT, specifically a disruption in their inosinate cycle that leads to a high purine production and a number of other metabolic abnormalties (*44*). LN cells infected with viral particles containing the HPRT vector could be rescued in selective medium. Seventeen HPRT$^+$ clones were isolated and studied. These cell lines had HPRT levels ranging from 4 to 23 percent of the normal level, and the abnormalities associated with a deranged inosinate cycle were partially to nearly completely corrected (*43*). In a corollary study, viral particles containing the HPRT-vector were used to infect mouse bone marrow cells that were then injected into lethally irradiated mice (*19*). Both human HPRT proteins and chronic production of HPRT-vector particles were detected in the hematopoietic tissue of the mice.

A problem must still be overcome, however. Even though expression of HPRT and *neo*^{r} genes has been obtained in the hematopoietic tissue of irradiated mice, the efficiency of the combined delivery-expression system is poor. If 15 percent of stem cells can be infected and if 4 to 23 percent of normal expression can be obtained in them, can sufficient enzyme be synthesized to be of benefit to a patient? The issue, once again, is whether or not the treated cells will have a selective growth advantage in the patient's body. If they do not, then, either the patient's own bone marrow must be partially or totally eliminated before reimplantation of the treated cells or the gene therapy protocol must demonstrate at least some expression in nonirradiated animals. It must be recognized, however, that, in the absence of a true animal model for a given genetic disease, it might be difficult or impossible to demonstrate selective growth advantage except in human patients.

Use of enhancers to increase expression. How can the level of expression be increased and properly regulated? One key element may be the enhancers. These are DNA sequences usually 50 to 150 base pairs in length that increase the expression of the adjacent gene 10 to 1000 times (*45*). A retrovirus has its own enhancer immediately upstream from its promoter in the LTR (Fig. 1). Enhancers are known to be species-specific (*46*). A primate enhancer (for example, the 72 base pair repeat from SV40) is several times more active in primate tissue culture cells than in rodent cells. Likewise, a mouse enhancer (for example, the 73 base pair repeat from MSV) is more active in rodent cells than in primate cells. The promoter acted upon does not influence the species specificity (a mouse β-globin promoter and a primate SV40 promoter are both activated more by a primate enhancer in primate cells than in rodent cells), although different promoters can be enhanced to different extents (*47*). Retroviral vectors designed for therapeutic application in humans may need primate, or even human, enhancing sequences rather than the mouse ones that are now used.

Some enhancers may even be tissue-specific (*48*). With a tissue-specific enhancer it may not be necessary to develop a cell-specific delivery system. The DNA could be integrated into all cells but only be expressed significantly in that tissue in which the enhancer is active. Even more precision may be achieved if one could place a tissue-specific coat on a retroviral particle that would direct the virus into the target cell, along with a tissue-specific (and possibly even a developmental-time-period-specific) enhancer in the construct itself.

Systems like globin undoubtedly have other regulatory regions in addition to enhancers which recognize cellular factors that are involved in control. Much information still needs to be learned about the regulatory signals in these multigene families.

Expression from plasmid-based expression vectors. If a chemical gene transfer technique is used as a delivery system, then the gene must be inserted into an appropriate expression vector. An expression vector is a plasmid (usually pBR322) in which the complementary DNA (or genomic gene) of interest is inserted together with regulatory signals. A typical expression vector would be composed of a promoter (for example, from the mouse metallothionein gene), the complementary DNA of choice, a splice site and polyadenylation site (necessary for correct processing of the transcribed RNA), and an enhancer.

Plasmid-based expression vectors containing an enhancer have not yet been used to transfect bone marrow cells. Therefore, how effective expression might be is unknown. The inefficiency of the presently available delivery systems for these vectors was discussed above.

One additional complication is that calcium phosphate–directed transfection, as well as microinjection, does not usually result in the integration of a single copy of the expression vector. The plasmid DNA vector appears to be ligated or replicated, or both, inside the cell to form a long head-to-tail structure called a tandem repeat (*49*). This tandem repeat, which can be a few or up to hundreds of copies in length, is randomly inserted usually in one site in the genome. The tandem repeats may produce problems for genes requiring intricate regulation because of the uncertainty as to how many of the copies are active.

Regulation by genomic control signals. Can either plasmid-based expression vectors or retroviral vectors be used to transfer genes that are controlled by the gene's own genomic regulatory sequences? Plasmid-based expression vectors in transgenic mice do respond to normal physiological control signals in some cases. Metallothionein-promoted genes express primarily in the liver, the

normal tissue for metallothionein synthesis, and can be induced by cadmium, as occurs in vivo for the endogenous gene; however, they do not respond to steroids, which are another physiologic inducer in vivo (*50*). An immunoglobulin gene is expressed in the spleen, the correct in vivo tissue, and not in liver (*35*). A mouse-human β-globin fusion gene expresses in hematopoietic tissue (*51*).

In tissue culture cells, a number of plasmid-based expression vectors have demonstrated at least a degree of normal regulation. For example, the human β-globin gene with approximately 1 kilobase of genomic 5′ flanking sequence can be induced (along with endogenous mouse globin) in a transfected MEL cell (*52*). The level of expression is not as high as that of the normal endogenous β-globin gene, suggesting that other regulatory signals are needed. However, transfection of MEL cells with cosmids carrying 30 to 40 kilobases of human genomic DNA containing the human β-globin gene does not result in higher expression of human β-globin messenger RNA (*53*).

Miller *et al.* (*54*) obtained encouraging results when they placed a rat growth hormone complementary DNA together with 237 bases of genomic 5′ flanking sequence into the *env* region of the HPRT-vector already described above. This growth hormone gene was regulated in rescued HPRT$^-$ fibroblast cells by its own genomic promoter and regulatory sequences as shown by (i) stimulation by glucocorticoid and thyroid hormones, which are normal in vivo regulators, and (ii) equal activity whether the fragment was placed in the same direction or opposite to the vector's LTR's (*54*). Expression of the vector in an animal has not yet been studied.

These data provide hope that vectors can be built with all the genomic regulatory signals necessary to produce correctly controlled expression in target cells. In the future, one might use only selected portions of a retrovirus in order to construct a delivery and integration system that would place one copy of the vector DNA into the target cell's genome. Expression would be controlled by the exogenous gene's own genomic regulatory signals. One possible problem is size: it appears that MoMLV constructs must not be over 9 to 12 kilobases in order to be packaged. Since 2 or 3 kilobases are necessary for essential function, only 6 to 9 kilobases are available for insert. This amount may be adequate, but further studies are needed to determine the answer (*55*).

Importance of chromosomal location. A major question that remains is: How important is chromosome location? Integration of a proviral structure can in some cases activate a downstream gene, as can occur with oncogenes. This problem could be eliminated by deleting the enhancer and promoter regions from the 3′ (right-hand) LTR in the retroviral vector. One round of reverse transcription could then occur which would result in double-stranded retroviral DNA with both LTR's defective. The retroviral vector DNA would then integrate with no transcription initiation signals. Therefore, expression would have to be controlled by exogenous signals in the inserted gene, and no downstream activation of other genes could take place.

Certainly an integration site that disrupts an important gene or regulatory sequence would normally be detrimental. How often this would occur must be determined by experiment. It is probable though that in most such cases the insertional event would diminish the fitness of the recipient cell so that it would be outgrown by normal cells.

Are there only certain active chromatin regions that can allow expression of a gene? Or could an expression vector take its own "active domain" with it so that essentially any location would be acceptable? The answers to these questions are still not known.

Safety

The third and final criterion for evaluating a human gene therapy protocol is that the delivery-expression system be safe.

Retroviral vectors. Although retroviruses have many advantages for gene transfer, they also have disadvantages. One problem is that they can rearrange their own structure as well as exchange sequences with other retroviruses. In the future it might be possible to modify retroviral vectors in such a way that they become less unstable. At present, however, there is the possibility that a retroviral vector might recombine with an endogenous viral sequence (*56*) to produce packageable, infectious recombinant virus. Properties that such a recombinant would have are unknown, but the potential homology between retroviral vectors and as-yet unknown primate cancer retroviruses or human T-cell leukemia viruses might be sufficiently close so that possible recombinants should be sought. There is, however, a built-in safety feature with the mouse retroviral vectors now in use. These mouse structures have a very different sequence from known primate retroviruses, and there appears to be little or no homology between the two (*57*). Therefore, a potentially "safe" proviral vector construct might be one composed of mouse LTR's, with their enhancer and promoter regions deleted, and a human gene controlled by the appropriate human genomic regulatory signals.

With the present constructs, three types of experiments ought to be carried out before any retrovirus-treated bone marrow is injected into a patient. These protocols, designed to test the safety of the delivery-expression system, are necessary since once treated bone marrow is reinserted into a patient, it and all retroviruses that it contains are irretrievable.

First, studies in vitro with human bone marrow are needed. Marrow cultures infected with the therapeutic vector should be tested for a period of time for the production of recombinant viruses. Any infectious virus isolated should be studied for possible pathogenicity.

Second, studies in vivo with mice are needed. Since many retroviral vectors are constructed from mouse retroviruses, and expression studied in mouse bone marrow transplanted into lethally irradiated (or nonirradiated) mice, these animals should be followed to determine if genomic rearrangement or the site of chromosomal integration has resulted in any pathologic manifestations or the production of any infectious viruses.

Third, studies in vivo with primates are needed. A protocol similar to the one planned for human application should be carried out in primates, not just mice, because the endogenous proviral sequences in primate, including human, DNA are different from those in mouse DNA. Therefore, the nature of any viral recombinants would be different. Treated bone marrow should be reimplanted into primates, the successful transfer of intact vector DNA into hematopoietic cells demonstrated, the expression of at least small amounts of gene product verified, and the existence of infectious recombinant viruses sought and, if found, analyzed.

Plasmid-based expression vectors. The calcium-phosphate procedure for transferring a plasmid-based expression vector into human bone marrow has not yet been demonstrated to be an effective delivery system. However, the procedure itself does not appear to represent a significant risk of harm. In theory, of course, a stem cell could be altered to make it carcinogenic so that it would still be necessary to follow treated mice over time to determine the likelihood of pathology. Primate studies, however, would appear not to be necessary.

Review Procedure

The initial clinical protocols designed to carry out gene therapy in patients will probably be evaluated in the following way. Under current Department of Health and Human Services regulations for the protection of human research subjects, a human gene therapy protocol must be reviewed by the Institutional Review Board at the investigator's home institution. In addition, because of the widespread public interest and concern in this area, the National Institutes of Health has announced (*58*) that any federally funded gene therapy experiment involving recombinant DNA must first be approved by the NIH after review by the Recombinant DNA Advisory Committee (RAC). Prior to review by RAC, proposals will be examined by a special RAC working group on human gene therapy (*59*). In addition, the Food and Drug Administration could regulate the DNA used in a human trial as a biological drug, analogous to polynucleotide interferon inducers, interferons, and vaccines (*60*). The Food and Drug Administration is currently exploring its regulatory responsibilities in this area (*61*).

Representative Albert Gore's proposal for a President's Commission on the Human Applications of Genetic Engineering (*62*) has just passed both houses of Congress in a modified form. This commission, if signed into law, would probably concern itself primarily with matters of policy and procedure rather than the review of individual recombinant DNA research proposals (*63*); the initial protocols, however, might be of particular interest to the commission in helping it to define the scope of its efforts.

Conclusion

It now appears that effective delivery-expression systems are becoming available that will allow reasonable attempts at human gene therapy. These systems are based on treatment of bone marrow cells with retroviral-vectors carrying a normal gene. The safety of the procedures is the remaining major issue. Patients severely debilitated by being homozygous for a defect in the gene for one of the enzymes HPRT, PNP, or ADA are the most likely first candidates for gene therapy.

It is unrealistic to expect a complete cure from the initial attempts at gene therapy. Many patients who suffer from severe genetic diseases, as well as their families, are eager to participate in early clinical trials even if the likelihood is low that the original experiments will alleviate symptoms. However, for the protection of the patients, particularly since those with the most severe diseases and, therefore, the most ethically justifiable first candidates, are children, gene therapy trials should not be attempted until there are good animal data to suggest that some amelioration of the biochemical defect is likely. Then it would be necessary to weigh the potential risks to the patient, including the possibility of producing a pathologic virus or a malignancy, against the anticipated benefits to be gained from the functional gene. This risk to benefit determination, a standard procedure for all clinical research protocols, would need to be carried out for each patient.

In summary, institutional review boards should carefully evaluate therapeutic protocols to ensure that the delivery system is effective, that sufficient expression can be obtained in bone marrow cultures and in laboratory animals to predict probable benefit, even if small, for the patient, and that safety protocols have demonstrated that the probability is low for the production of either a malignant cell or a harmful infectious retrovirus. Once these criteria are met, I believe that it would be unethical to delay human trials. Patients with serious genetic diseases have little other hope at present for alleviation of their medical problems. The issues of germ line therapy and enhancement engineering need to be debated widely in society, but arguments that genetic engineering might someday be misused do not justify the needless perpetuation of human suffering that would result from an unnecessary delay in the clinical application of this potentially powerful therapeutic procedure (*64*).

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Exhibit 4

Gene Therapy

Treatment of disease by introducing healthy genes into the body is becoming feasible. But the therapy will not reach its full potential until the genes can be coaxed to work throughout life

by Inder M. Verma

One infant in every hundred is born with a serious genetic defect. Usually the damage becomes evident in childhood. All too often, it gives rise to physical or mental abnormalities, pain and early death. Of the more than 4,000 known inherited disorders, most lack fully effective therapies.

It is no wonder, then, that scientists have long imagined curing heritable ills by introducing healthy genes into patients. Advances in recombinant DNA technology, which have made possible the isolation of many genes, and new insights into gene regulation are beginning to make this once impossible notion seem feasible.

Indeed, the first federally approved clinical trial of a gene therapy for a genetic disease began this past September. R. Michael Blaese, W. French Anderson and their colleagues at the National Institutes of Health (NIH) are introducing the gene for the enzyme adenosine deaminase (ADA) into children suffering from a rare condition known as severe combined immunodeficiency (SCID). Derangement of this gene debilitates the immune system and is responsible for about 25 percent of all cases of SCID.

The approach of the NIH group requires repeated treatments throughout life, and so it is not a cure. Still, the trial could represent the start of a new era in medicine. The current pace of research suggests that by the turn of the next century clinical trials of gene therapies may be under way for any of a number of diseases—inherited and otherwise.

Genes can be transferred either into germ cells (sperm, eggs or early embryos) or somatic cells (those not destined to become sperm or eggs). Yet germ-line therapy is not an option for the foreseeable future, in part because the new genes would be passed from generation to generation, a prospect that raises profound ethical concerns.

For instance, should therapy be applied simply to improve one's offspring, not only to prevent an inherited disease? Who would be empowered to decide? Is society willing to risk introducing changes into the gene pool that may ultimately prove detrimental to the species? Do we have the right to tamper with human evolution? The prospect of somatic cell therapy is less troubling, mainly because it would affect only the treated patient.

The most promising candidates for somatic cell therapy are disorders caused by impairment of a single gene that has been isolated and cloned and so is available for transplant. These diseases should be simpler to correct than those caused by multiple genes or by such global disturbances as the loss or addition of whole chromosomes. (Normally, human cells carry one set of 23 chromosomes inherited from the mother and a corresponding set from the father. Every chromosome consists of a long stretch of DNA and includes thousands of genes.)

In the ideal world, the diseases would be cured for life by one treatment, with no side effects. And gene insertion into a chromosome in a target somatic cell would be site specific: in what is called homologous recombination, the healthy, or "therapeutic," gene would exactly replace the damaged copy. Targeted insertion increases the probability that a therapeutic gene will function correctly. It also reduces the likelihood that random insertion will activate a quiescent oncogene (a cancer inducer) or inactivate a cancer suppressor.

In reality, investigators have found it extremely difficult to control the fate of DNA introduced into cells. For every gene spliced into the correct place, more than 1,000 fit randomly into the genome (the total DNA in a cell). Work by Mario R. Capecchi of the University of Utah suggests that the obstacles to site-specific gene delivery are great but surmountable. Meanwhile many laboratories, including my own at the Salk Institute in La Jolla, Calif., are concentrating on developing gene augmentation therapy, in which a healthy gene replaces the product of a missing or defective gene but does not physically replace the flawed DNA itself.

Augmentation can be helpful when a genetic derangement results in little or no production of a protein. (Each gene encodes, or carries instructions for, a single protein.) Low production occurs when mutations hamper the activity of both the maternal and paternal copies of a gene or when a hobbled gene is inherited on a male's only X chromosome. (The cells of males carry one X and one Y chromosome; those of females carry two X chromosomes.)

On the other hand, augmentation therapy might not be of much help when a mutation yields overproduction of a protein or the synthesis of a destructive substance, as is the case in sickle cell anemia. To correct those kinds of disturbances, therapy would often have to include delivery of both a healthy gene and one capable of inactivating the mutated version.

For now, most scientists interested in gene augmentation are planning to remove cells from patients, introduce a therapeutic gene and return the altered cells to the subject. Some day, however, physicians may directly inject patients with genes linked to substances that will deliver those genes to specific target cells.

Fortunately, genetic flaws do not necessarily have to be corrected in all of

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the body's millions of cells in order for therapy to work. First, even though every somatic cell in an individual carries identical chromosomes, certain genes function only in a single cell type. Treatment, then, could focus only on that type. Second, even when a genetic defect results in insufficient synthesis of a protein made in virtually every cell, many cells compensate for the loss. For instance, a flaw in the ADA gene affects most somatic cells to a degree but is devastating only to some constituents of the immune system.

Nontargeted delivery of genes into cells can be accomplished by chemical or physical means (transfection) or by viruses (transduction). In chemical approaches, one mixes many copies of DNA carrying the healthy gene with a charged substance—typically calcium phosphate, DEAE-dextran or certain lipids. Then the mixture is essentially dumped onto recipient cells. The chemicals disturb the cell membrane and transport the DNA into the interior.

The procedure is simple, but the efficiency of gene delivery is dismal. Usually only one cell in 1,000 to 100,000 integrates the gene of interest into its genome. A physician would have to obtain an impossible number of cells from patients to guarantee the appropriate alteration of the millions required for therapy.

I should point out that integration is not always crucial to gene expression (production of the encoded protein). Still, a gene that is integrated is likely to last longer in the cell. Further, it should replicate whenever the rest of the DNA does, as when a cell prepares to divide. The therapeutic gene would thus be inherited by the daughter cells and by their daughters and so on, thereby ensuring a supply of the product throughout a patient's life.

Physical methods include microinjection with a fine glass pipette and electroporation (the exposure of cells to an electric shock). The shock renders cells permeable to DNA in the surrounding medium, but it can also severely damage them. Microinjection can be extremely efficient; perhaps one cell in five takes up the foreign gene permanently. Yet because only a single cell can be injected at a time, this tedious, labor-intensive approach is not suitable for therapeutic purposes.

The final strategy capitalizes on the native ability of viruses to enter cells, bringing their own genetic material with them. Many of these organisms have now been engineered to serve as vectors, or delivery vehicles, for gene transfer. Viruses can be grouped according to whether their genetic material is DNA or RNA. The two substances have important chemical differences, although both are built from units known as nucleotides and both include regulatory codes in addition to those specifying the sequences of amino acids in proteins.

Many DNA viruses that can accept foreign genetic material turn out to be severely limited in the number of nucleotides they can accommodate and in the range of cells they infect. Certain other DNA viruses are roomier but have so far proved unusable for ver-

STERILE BUBBLE protected a boy named David, who suffered in the 1970s from severe combined immunodeficiency, or SCID, an inherited disorder in which the immune system is profoundly impaired. SCID patients have better options today and may have more in the future: the first gene therapy approved for clinical trial aims to ease a form of the disorder.

ious reasons. Moreover, DNA viruses often do not splice their genetic material into the chromosomes of the cells they infect.

As is true of the DNA viruses, most RNA viruses are unsuitable for gene therapy, mainly because RNA, which cannot integrate into the DNA of human cells, is degraded rapidly. Varieties known as retroviruses are an exception. They actually convert their RNA to DNA in infected cells and insinuate the DNA into a chromosome. The integrated DNA then directs the synthesis of viral proteins. Retroviruses can entertain more foreign genetic material than some DNA viruses. They can also infect a broad spectrum of species and cell types.

For these reasons, retroviruses are the most promising gene-delivery systems studied thus far. Indeed, unless specified, all approaches to gene transfer discussed in the balance of this article are based on these vectors.

Retroviruses are, of course, not without obvious drawbacks. For instance, they can merge their DNA into a chromosome only in cells capable of actively dividing. Yet many cells do not normally divide—among them, mature neurons—and so they are not readily amenable to being genetically altered by retroviral vectors.

More disturbing is the possibility that retroviruses can cause cancer. The risk is extremely low for the species that have been considered as vectors, but it increases if the viruses are allowed to multiply in the body and spread from cell to cell. Consequently, a major challenge has been devising ways to stop the vectors from reproducing.

The efforts of several laboratories have together yielded at least one technique that seems to work well [*see illustration on page 72*]. The organisms produced by that method have a normal outer coat and contain all of the virus's proteins. The retroviral RNA, however, includes no instructions for synthesizing viral proteins. The therapeutic gene takes the place of those missing instructions.

The coat enables the viruses to enter cells and deliver the viral contents to the cell's cytoplasm. Then viral enzymes convert the RNA to DNA and help to fit that DNA into the genome of the host cell. But that is the end of the line for the virus.

Under normal circumstances, integrated retroviral DNA—called the provirus—would direct the synthesis of viral proteins and RNA, which would then assemble into clones of the original virus. In contrast, the altered retrovirus, bereft of instructions for making viral proteins, produces no progeny. The virus essentially disappears from the cell, leaving behind only the foreign gene and nucleotide sequences that now serve merely to facilitate the expression of the gene.

Although retroviruses can infect many cell types, only certain target cells can be considered for genetic manipulation. The cells must be strong enough to withstand handling and capable of being removed from the body and returned with reasonable ease. In addition, they should be long-lived, surviving for months or years or preferably for the patient's entire life. Because bone marrow, skin and liver cells best meet these criteria, diseases that can be treated by manipulating these cells are among the most promising candidates for gene therapy.

The cells of the bone marrow, where blood is produced, can in theory be exploited to correct disorders caused by genetic flaws in red blood cells or in white blood cells (which are important in immunity). SCID caused by an ADA deficiency is but one of several inherited conditions affecting the immune cells; another is leukocyte adhesion deficiency, which involves the poor mobilization of white blood cells and leads to recurrent infections. Among the diseases associated with impaired red blood cells are the thalassemias, which reflect impairments in the genes encoding subunits of the hemoglobin molecule—the oxygen carrier in red blood cells.

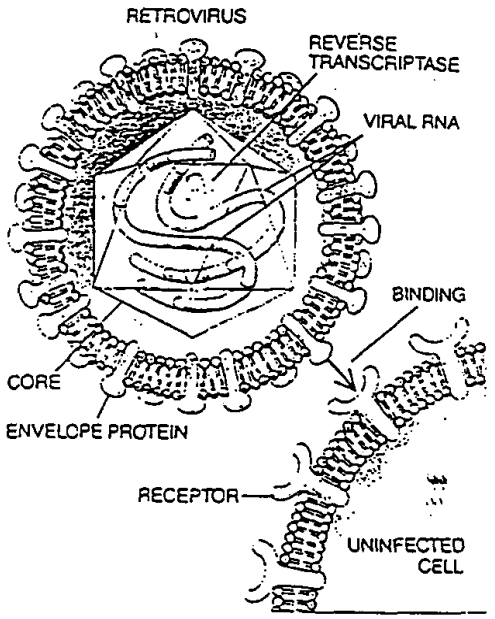
Beta thalassemia was once expected to be the first disorder treated with gene therapy. Its history illustrates some of the problems that have beset the effort to develop gene therapy in general and therapy based on bone marrow cells in particular.

Red blood cells of patients stricken with beta thalassemia are deficient in beta globin, which in healthy individuals combines with alpha globin and iron (heme) to yield hemoglobin. Healthy cells regulate the activity of both genes precisely, ensuring that equal amounts of alpha and beta globin are made. The lack of beta globin gives rise not only to a deficit in hemoglobin production but also to a relative excess of alpha globin. This excess, in turn, hastens cell death and can cause severe anemia. Usually patients succumb to the disease by age 20, after years of pain and suffering.

This disease and other inherited blood disorders could probably be treated efficiently by delivering healthy genes to stem cells, the subset of cells in the marrow that gives rise to the full spectrum of blood cells and replaces dead cells throughout a person's life. Stable introduction of a desired gene into a stem cell could guarantee the production of normal blood cells for as long as a patient lives.

Sadly, human stem cells are far from abundant and are virtually impossible to isolate. Researchers have therefore been forced to resort to a less efficient strategy: infecting enormous numbers of bone marrow cells with a therapeutic retrovirus in the hope that enough stem cells will be infected.

Studies of beta globin have supplied much of the evidence showing that the approach has at least some merit.



LIFE CYCLE of a retrovirus begins when the virus binds to (*above*) and enters (*right*) a cell and injects its genetic material (RNA) and proteins into the cytoplasm. Typical retroviral RNA includes three coding regions: *gag* (*green*), *pol* (*blue*) and *env* (*purple*), specifying, respectively, proteins of the viral core, the enzyme reverse transcriptase and constituents of the coat. It also has three noncoding domains—two at the tips (*light orange*) and another called psi, ψ (*red*). In the cytoplasm, reverse transcriptase converts the RNA into DNA, whose lengthened terminal domains, called long-terminal repeats (*dark orange*), influence the activity of viral genes and facilitate insertion of viral DNA into cellular DNA. The ensconced DNA (the provirus) directs the synthesis of viral proteins and RNA. The proteins then enclose the RNA, forming viral particles that bud from the cell.

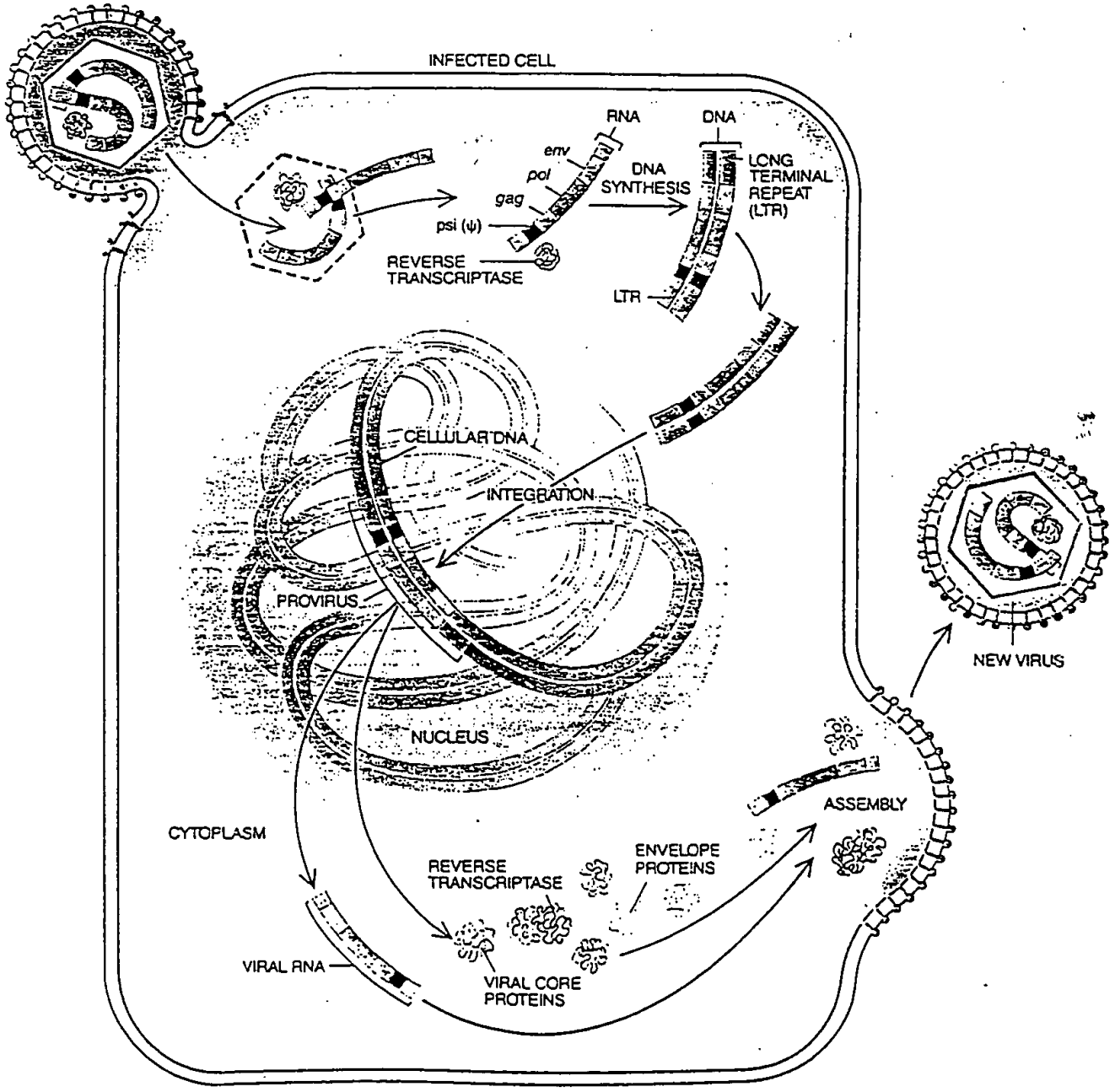
For instance, several laboratories have shown that a human beta globin gene inserted into mouse bone marrow cells by retroviral vectors stays in the cells. And Richard C. Mulligan and his coworkers at the Whitehead Institute for Biomedical Research in Cambridge, Mass., have further shown that the human gene is expressed when such cells are implanted in mice.

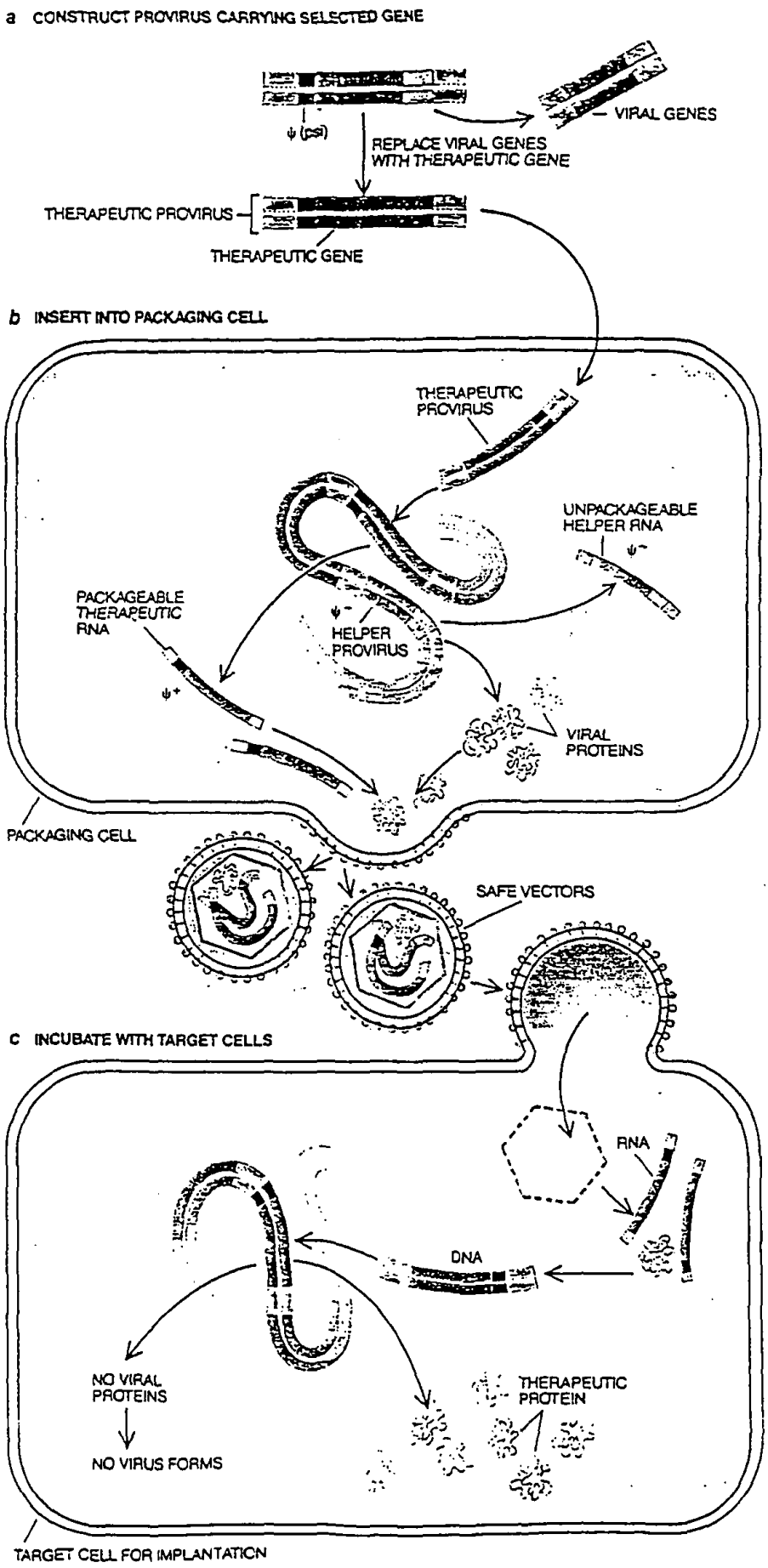
On the other hand, no one has been able to achieve significant levels of globin synthesis in recipient animals. This problem has been a major disappointment, but a discovery by F. G. Grosveld and his colleagues at the National Institute for Medical Research in London offers hope for a solution.

They identified distinct stretches of DNA, thousands of nucleotides apart from the gene itself, that in normal red blood cells dramatically boost the production of globin messenger RNA. Messenger RNA is transcribed, or copied, from DNA and is the template from which protein is made; hence, high levels of a messenger RNA indicate that the encoded protein is being produced in abundance. It seems reasonable to think that linking globin-specific enhancers to a globin gene in a retroviral vector might enhance globin synthesis in the body. Studies of this hypothesis are in progress.

In general, genetically altered bone marrow cells have yielded poor in vivo expression of other genes as well. The problem must be resolved before gene therapy based on bone marrow cells can become a reality.

Along with an acceptable level of gene expression, one would hope for long-term activity. Recent findings relating to globin indicate that achieving prolonged expression of genes inserted in bone marrow may be less problematic than attaining high levels of protein synthesis. For instance, Chung L. Li and V. J. Dwarki in my laboratory have produced sustained, albeit weak, expression of the human beta globin gene in mice for at least a five-month study period—the equivalent of 15 to 20 years in a human being. The alpha





globin gene remained functional for at least 10 months.

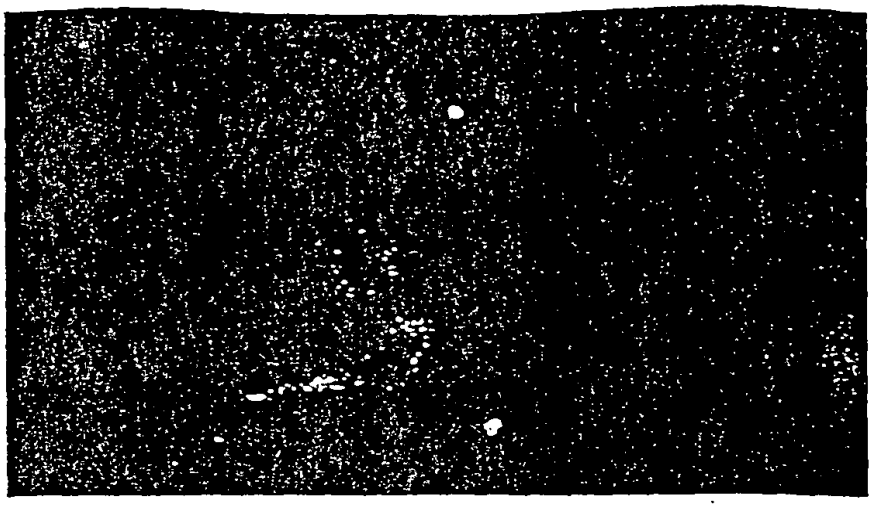
Other findings emerging from the work on beta thalassemia highlight the complexity introduced when correction of a disease requires precisely regulated expression of a therapeutic gene. For many disorders, including SCID, simply producing some amount of a missing protein is better than none. The same is not true for thalassemia. Because a relative excess of either alpha or beta globin can damage cells, the activity of a therapeutic globin gene must exactly mimic that of a normal version. Unfortunately, the mechanisms that control the activity of genes are understood only imperfectly—both for the beta globin gene and for most others. Discoveries are made constantly, however, and are helping improve the design of vectors for gene therapy.

SCID researchers at the NIH have taken a detour from gene therapy based on bone marrow cells, in part because of the ongoing problem of poor expression. Patients in their study are treated with a select subset of circulating *T* lymphocytes, white blood cells crucial to immunity. *T* cells are devastated by a lack of ADA.

The retrovirally altered lymphocytes are infused into children who are now being helped somewhat by injections of PEG-ADA—ADA mixed with the chemical polyethylene glycol to increase the enzyme's half-life. Success of the approach will be measured by improvements in immune function beyond that achieved by enzyme replacement alone. Regrettably, *T* cells do not have the longevity of stem cells, which is why the disease cannot be cured indefinitely by one treatment.

RETROVIRAL VECTORS are assembled, or packaged, in cells designed to release only safe vectors. Investigators substitute a therapeutic gene for viral genes in a provirus (*a*) and insert that provirus into a packaging cell (*b*). The viral DNA directs the synthesis of viral RNA but, lacking viral genes, cannot give rise to the proteins needed to package the RNA into particles for delivery to other cells. The missing proteins are supplied by a "helper" provirus from which the psi region has been deleted. Psi is crucial to the inclusion of RNA in viral particles; without it, no virus carrying helper RNA can form. The particles that escape the cell, then, carry therapeutic RNA and no viral genes. They can enter other cells (*c*) and splice the therapeutic gene into cellular DNA, but they cannot reproduce.

The availability of nongenetic treatments for SCID (including bone marrow transplantation) raises the general question of whether subjecting patients to highly experimental gene therapies is justified when alternatives exist. The prevailing opinion holds that such experimentation is acceptable if the risks are demonstrably low and if, on the one hand, a gene therapy promises to be significantly more helpful than existing approaches or, on the other, patients are ineligible for the established treatments. In the case of SCID, for example, not all patients have access to bone marrow from a tissue-compatible donor.



LIVER CELLS from rabbits genetically deficient in the receptor for low-density lipoprotein (LDL) began to make the missing receptor (*bright regions*) after being altered to carry the receptor gene. The finding raises the possibility that a similar genetic disorder leading to excess serum cholesterol in humans might one day be treatable by gene therapy. James M. Wilson of the Howard Hughes Medical Institute Research Laboratories at the University of Michigan at Ann Arbor and J. Roy-Chowdhury of the Albert Einstein College of Medicine made the photomicrograph.

Genetic alteration of lymphocytes or bone marrow cells aims to correct defects in those same cells or their progeny. Skin cells, in contrast, are being studied for quite a different purpose: the synthesis and secretion of proteins that are normally made in one cell type but are ferried in blood plasma for use by other cells.

In principle, implants of skin cells could correct many disorders. These conditions might include hemophilia (caused by a lack of blood-clotting factors made in the liver) and diseases caused by insufficient production of particular hormones (for example, growth hormone). Certain disorders caused by deficient production of widely made proteins would also be candidates, if the tissues most affected by the deficiency could take up replacement proteins from the blood.

Fibroblasts, a constituent of the dermis (the lower layer of the skin), are best suited for therapy, which would involve implanting the altered cells back into the dermis. They are accessible and strong and able to multiply in the laboratory. Furthermore, they can secrete substances into the blood and would be easy to remove if necessary.

My laboratory has extensively studied the value of skin fibroblasts for treating the form of hemophilia caused by a lack of the liver product known as clotting factor IX. Our results underscore the great therapeutic potential of such cells.

In one of our studies, for instance, A. Dusty Miller, now at the Fred Hutchison Cancer Research Center in Seattle, collaborating with George G. Brownlee and Don S. Anson of the University of Oxford, showed that fibroblasts could be induced to synthesize and secrete factor IX, even though they do not typically make that protein. (Whether the same will be true for all foreign proteins remains to be seen.) Furthermore, when Daniel C. St. Louis, Jonathan H. Axelrod and Raphael Scharfmann in my group used retroviruses to insert the human factor IX gene into fibroblasts and implanted the cells in the dermis of mice, the implants became highly vascularized and released the factor into the blood.

This study not only demonstrated that expression of factor IX in animals was possible, it also taught us an important lesson. About 15 days after the cells were implanted, the human factor disappeared from the blood of the mice. The recipients, it turned out, had mounted an immune response against the foreign human protein. The moral: gene therapy will probably be most successful in patients who make at least a small amount of a deficient protein; otherwise the immune system may become aroused against the product of an inserted gene.

We have also found some evidence to suggest that, unlike the bone marrow cells studied to date, fibroblasts may be able to produce enough of a selected product to correct disease. Extrapolation from data in mice indicates that an implant the size of a quarter should make enough protein to alleviate a factor IX deficiency in a human. In collaboration with Kenneth M. Brinkhous of the University of North Carolina at Chapel Hill, we expect to study the ability of fibroblast implants to correct hemophilia in dogs. If those experiments are successful, trials in humans would be justified.

Genetically altered fibroblasts might also be implanted in the brain to correct disorders in neurons. The brain is notoriously hard to treat because many drugs that circulate in the blood are barred from the brain. Moreover, neurons cannot be removed for direct genetic alteration without consequence to the brain. Fibroblasts could in theory be engineered to secrete proteins for diffusion into nerve cells.

Preliminary results are encouraging. Fred Gage of the University of California at San Diego has shown that implants engineered to secrete nerve growth factor could stimulate neuronal growth in the rat brain. The regeneration occurred in the kinds of neurons whose decay is associated with memory loss in Alzheimer's disease, although the role of the factor in that disease has not been established. Similarly, implants that make levodopa (L-dopa), a precursor of the neurotransmitter dopamine, are under study in animal models of Parkinson's disease. No one knows exactly what causes Parkinson's, but a deficiency of dopamine seems to play a part. Exactly how long fibroblast implants can survive in the skin or brain is still being investigated.

Compared with bone marrow and skin cells, liver cells are a newcomer to the field of gene therapy. They could become important for the treatment of any number of genetic diseases caused by malfunctioning liver cells. Recently, for instance, Mulligan of the Whitehead Institute and James M. Wilson, then also at the institute, and, separately, Theodore Friedmann and his colleagues at San Diego succeeded in delivering the gene for

the low-density lipoprotein (LDL) receptor to liver cells and inducing them to make biologically active receptors in the laboratory. The cells came from Watanabe rabbits, which are genetically deficient in the LDL receptor—as are humans afflicted with familial hypercholesterolemia, a condition that can lead to heart attacks.

The feasibility of directly injecting live Watanabe rabbits with complexes of the receptor gene and a protein that homes to the liver has also been studied. (Direct injection in humans would, of course, avoid surgery to remove liver cells.) The encoded protein was detected in the body but, as was also true in the cell-culture study, was made only transiently. Longevity may yet be improved; investigation of liver cells is still in its infancy.

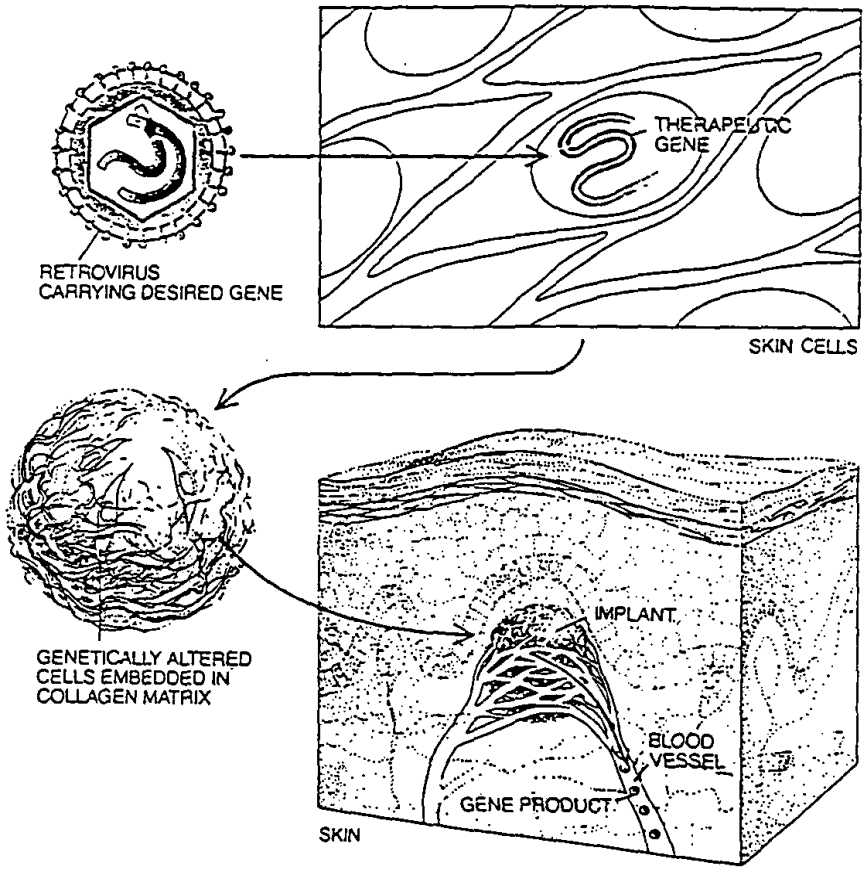
Although bone marrow, skin and liver cells are receiving the most attention, other types are also being considered. For instance, retroviruses can carry genes for secretory products into endothelial cells, which line the arteries. These cells have more intimate contact with the blood than do fibroblasts, and so they might deliver the products more quickly.

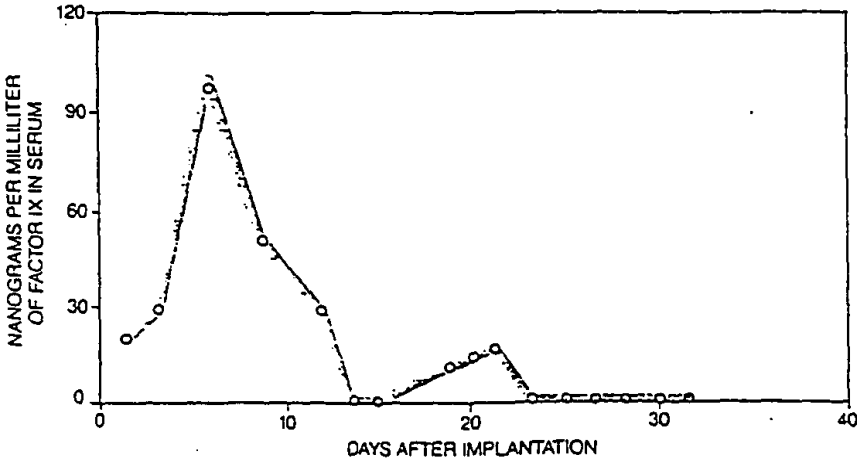
Researchers are also considering injecting a healthy gene encoding dystrophin (a structural component of muscle) directly into muscles of mice that have acquired a disorder akin to Duchenne's muscular dystrophy. There is reason to hope the genes will be expressed; other genes injected into muscles in live animals gave rise to proteins for several months, even though the DNA was not integrated into chromosomes. It may also be possible to treat cystic fibrosis, an inherited lung disorder, by packaging healthy genes in retroviruses that would be inhaled in an aerosol spray.

Gene therapy does not have to be limited to repairing the effects of malfunctioning genes. It can also add novel properties to cells to enhance their ability to combat disease.

For instance, Steven A. Rosenberg and his colleagues at the National Cancer Institute have demonstrated that lymphocytes taken from a patient's tumor and cultured with interleukin-2 (a *T* cell activator) can shrink some cancers. They now hope to increase the cancer-fighting powers of those tumor-infiltrating lymphocytes, or TILs, by inserting a gene encoding tumor necrosis factor, a potent immune-system molecule. The factor, which has anticancer activity, is not ordinarily made in *T* cells. Clinical trials are expected to begin soon [see "Adoptive Immunotherapy for Cancer," by Steven A. Rosenberg; SCIENTIFIC AMERICAN, May].

In more preliminary work, another group is trying to induce various cell types to produce CD4, a molecule found on *T* cells depleted by the AIDS virus. The virus enters the cells after a protein in its coat binds with CD4. A flood of CD4 molecules in the blood might serve as a decoy to keep the virus from interacting with the cells. Many other creative ideas for applying gene therapy are also being discussed, including coaxing endothelial cells to secrete factors that would pre-





IN CELLS carrying an inserted gene can be embedded in a collagen matrix and planted in the dermis to deliver the gene's product to the blood (*top*). In one experiment, skin fibroblasts containing the human gene for factor IX, a protein normally secreted by the liver to aid in blood clotting, became well vascularized in mice and secreted the human factor for approximately two weeks (*graph*). Much greater release of foreign proteins has now been achieved by fibroblast implants.

DISORDER	INCIDENCE	NORMAL PRODUCT OF DEFECTIVE GENE	TARGET CELLS	STATUS
Hemoglobinopathies (thalassemias)	1 in 600 in certain ethnic groups	Constituents of hemoglobin	Bone marrow cells (which give rise to circulating blood)	Globin production in animals receiving gene needs to be improved
Severe combined immunodeficiency (SCID)	Rare	Adenosine deaminase (ADA) in about a quarter of SCID patients	Bone marrow cells or T lymphocytes	Clinical trial of lymphocyte therapy for ADA deficiency is under way
Hemophilia A Hemophilia B	1 in 10,000 males 1 in 30,000 males	Blood-clotting factor VIII Blood-clotting factor IX	Liver cells or fibroblasts	Good chance for clinical trials (with fibroblasts) in next five years
Familial hypercholesterolemia	1 in 500	Liver receptor for low-density lipoprotein (LDL)	Liver cells	Animal studies are in early stages
Inherited emphysema	1 in 3,500	Alpha$_1$-antitrypsin (liver product that protects lungs from enzymatic degradation)	Lung or liver cells	Work is very preliminary
Cystic fibrosis	1 in 2,500 Caucasians	Substance important for keeping air tubes in lungs free of mucus	Lung cells	Aerosol delivery of gene directly to lungs is a theoretical possibility
Duchenne's muscular dystrophy	1 in 10,000 males	Dystrophin (structural component of muscle)	Muscle cells (particularly embryonic ones that develop into muscle fibers)	Work is preliminary. Nondystrophin genes injected into muscle have directed synthesis of the encoded proteins
Lysosomal storage diseases	1 in 1,500 acquires some form	Enzymes that degrade complex molecules in intracellular compartments known as lysosomes	Vary, depending on disorder	Most diseases would require delivery of gene into brain cells (a difficult task) as well as into other cell types

POTENTIAL CANDIDATES for the earliest gene therapies will be disorders caused by defects in a single gene that has been cloned. In general, physicians will remove cells from a patient, insert a healthy gene and return the cells to the body.

vent blood clots from forming in a patient's arteries after heart surgery.

The idea of introducing genes to correct heritable and other disorders is nothing less than revolutionary. Perhaps that is one reason why the field has progressed somewhat more slowly than was once expected. Modern creatures are the products of millions of years of evolution. One cannot expect that the initial stabs at inserting genes into cells will yield normal, stable expression easily.

Yet to cure diseases, investigators must find ways to ensure that therapeutic genes are expressed well and persistently in the body. Continually emerging clues, such as the importance of including particular enhancers with some genes in retroviral vectors, are beginning to point the way. Also needed are better methods for returning genetically altered cells (such as liver cells) to the body, ways of extending the survival of implanted cells, and techniques for isolating human stem cells (to replace the bone marrow cells now being studied).

At the same time, the safety of retroviral vectors must be confirmed in extensive studies of both small and large animals, and efforts to incorporate added safeguards should continue. In spite of the advent of retroviral vectors that cannot replicate, there is still a chance they could cause cancer. Efforts to develop alternatives to retroviral vectors should be pursued further as well, as should research into site-specific gene delivery.

The goal of curing genetic diseases for life with a single, safe treatment is unquestionably worth the effort being put into it, but I must end with the reminder that gene therapy cannot correct all human disease. Most human afflictions are not genetic. They are environmental, caused by microbial infections that spread because of poor sanitation, polluted drinking water, malnutrition and other factors that are outside the scope of genetic engineering. Those diseases, too, deserve increased study.

FURTHER READING

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